PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 : C12Q 1/34, 1/25, C12N 9/78	A1	(11) International Publication Number: WO 00/40744
		(43) International Publication Date: 13 July 2000 (13.07.00)

(21) International Application Number: PCT/EP99/09936

(22) International Filing Date: 14 December 1999 (14.12.99)

(30) Priority Data:

198 57 868.7	15 December 1998 (15.12.98)	DE
199 42 174.9	3 September 1999 (03.09.99)	DE

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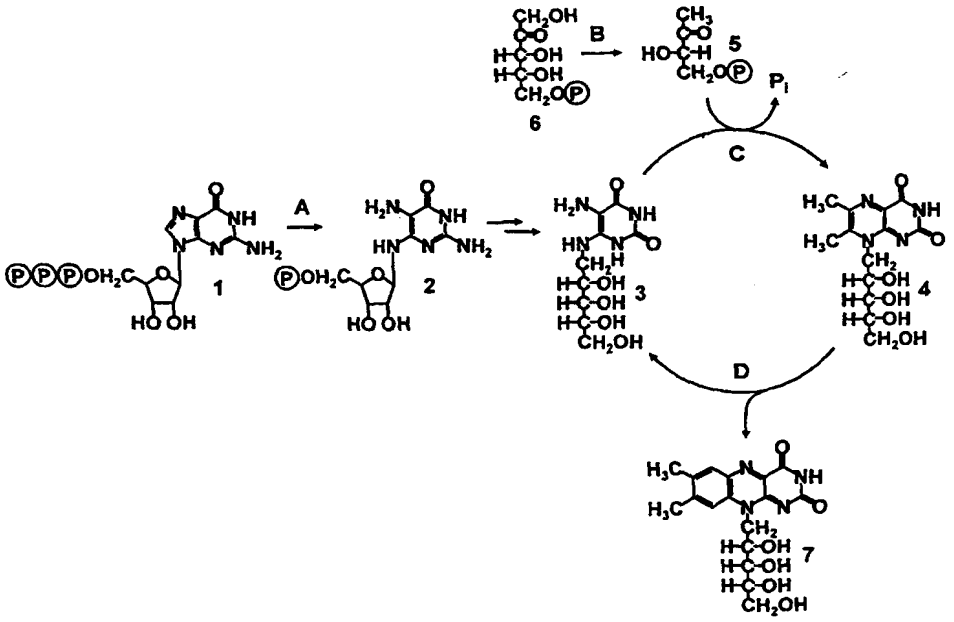
(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published

With international search report.

Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: METHOD FOR SCREENING FOR INHIBITORS OF RIBOFLAVIN BIOSYNTHESIS



(57) Abstract

Methods are described for screening for the presence or absence of inhibition of the activity of enzymes participating in riboflavin biosynthesis. GTP cyclohydrolase II activity is determined through the extent of conversion of GTP to 2,5–diamino–6–ribosylamino–4(3H)–pyrimidinone 5'–phosphate, catalysed by a protein having a GTP cyclohydrolase II sequence. The activity of 3,4–dihydroxy–2–butanone 4–phosphate synthase activity is detected through the extent of conversion of ribulose 5–phosphate to 3,4–dihydroxy–2–butanone 4–phosphate, catalysed by a protein having a 3,4–dihydroxy–2–butanone 4–phosphate synthase sequence.

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Method for screening for inhibitors of riboflavin biosynthesis

The present invention relates to the field of screening for inhibitors of riboflavin biosynthesis. Specifically, the invention relates to methods of screening for inhibitors of GTP cyclohydrolase II or 3,4-dihydroxy-2-butanone 4-phosphate synthase as well as for mutants that exhibit resistance to an inhibitor. The invention also relates to kits of parts for said screening methods. The invention relates further to the application of these screening methods for detecting inhibitors useful as herbicides, or fungicides or as antibacterial agents. The invention relates furthermore to plant enzymes for performing said screening methods.

The human population is situated in an ecology with an ever changing confrontation with dangerous organisms, in the agricultural area weeds, fungi or the like and in the medical or veterinarmedical area pathogenic bacteria, fungi or the like. There is a continued need to develop ever new chemical inhibitors against such undesirable organisms.

In times of a growing world population in conjunction with a growing awareness for the necessity of environmental protection there is a great need for increasing the productivity of agriculturally utilized land. One approach to pursue this problem in the search for novel herbicides or fungicides or the like exhibiting a high effectiveness against all pests such as weeds, fungi or the like, even when applied in small amounts, while not affecting crop plants as well as having little or no toxicity for man or mammals and preferably also all other animals in the relevant ecosystems.

In the medical or verterinary medical field there is an ever changing confrontation with pathogens, and notably pathogens with a significant propensity to develop resistance against conventional inhibitors. Inhibitors in this field should be highly effective against a pathogen and applicable in small amounts. They should show little or no toxicity for the patient or animal to be treated. And furthermore, they should be of such a nature that the product of the inhibited metabolic pathway should not be readily transported from the host organism through the cell wall of the pathogen. It is again apparent, that the detection of novel inhibitors satisfying the requirements is a formidable task.

One approach to finding novel inhibitors consists in the modification of known types of inhibitors. This approach promises effects which are not expected to be greatly dissimilar from the effects of the conventional members of these known types of inhibitors.

A more promising approach would be the search for novel types of inhibitors with novel mechanisms for effectiveness. Such a search cannot be guided by a knowledge of the structures of conventional inhibitors. Therefore novel methods of screening libraries of chemical compounds are required for this search.

It is therefore an object to provide a method for screening libraries of chemical test samples for compounds that inhibit with great effectiveness an enzyme in a biochemical pathway that is essential for weed plants, fungi or bacterial pathogens, but not for man or animals and whose inhibitors cannot be compensated by the uptake of the product of the pathway from the enviroment.

We have found that this object can be solved advantageously by a method for screening for inhibitors of riboflavin biosynthesis.

All cellular orgnisms require riboflavin as an indispensible requirement of numerous redox enzymes, many of which are crucial for the metabolism. All plants, fungi

and many bacteria generate riboflavin biosynthetically, whereas all animals require a nutritional source of riboflavin (Vitamin B_2). Therefore, an inhibitor for an enzyme in the biosynthesis of riboflavin in plants, fungi and bacteria would not interfere with the metabolism of animals. Furthermore, the absolute amount of riboflavin for cellular activity is low. Therefore, only small amounts of the enzymes of riboflavin biosynthesis are found in cells. This in turn means that only small amounts of an inhibitor for such an enzyme would be required. This makes the enzymes of riboflavin biosynthesis an ideal target for novel inhibitors.

The biosynthetic pathway of Vitamin B_2 (riboflavin) (Fig. 1) has been studied in considerable detail in bacteria and yeasts (for review see Bacher, 1991; Bacher *et al.*, 1996). The biosynthetic formation of one molecule of riboflavin (**7**) requires one molecule of GTP and two molecules of ribulose 5-phosphate. GTP (**1**) is initially converted to the committed product, 2,5-diamino-6-ribosylamino-4(3H)-pyrimidinone 5'-phosphate (**2**) by the enzyme, GTP cyclohydrolase II (step A). This intermediate is converted to 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione (**3**) by a sequence of deamination, side chain reduction, and dephosphorylation. The compound (**3**) is converted to 6,7-dimethyl-8-ribityllumazine (**4**) by condensation with 3,4-dihydroxy-2-butanone 4-phosphate (**5**) which is obtained enzymatically from ribulose 5-phosphate (**6**) by 3,4-dihydroxy-2-butanone 4-phosphate synthase (step B). The formation of (**5**) involves an unusual skeletal rearrangement with a loss of C-4 of the substrate as formate.

The *ribA* gene of *Bacillus subtilis* specifies a bifunctional protein catalyzing the formation of the pyrimidine (**2**) and of the carbohydrate (**5**) from GTP and ribulose-5-phosphate, respectively. Similar proteins have been predicted on the basis of DNA sequence information in several other microorganisms such as *Bacillus amyloliquefaciens*, *Synechocystis sp.*, *Mycobacterium tuberculosis*, *Actinobacillus pleuropneumoniae* or *Aquifex aeolicus (Gusarov et al., 1997; Kaneko et al., 1996; Cole et al. 1998 and 1996, Fuller et al., 1995; Deckert et al., 1998)*. On the other

hand, *Escherichia coli* and the yeasts *Saccharomyces cerevisiae*, *Schizosaccharomyces pompe, Candida guilliermondii, Azospirillum brasilense*, have been shown to specify GTP cyclohydrolases II and 3,4-dihydroxy-2-butanone 4-phosphate synthases as separate proteins specified by unlinked genes (*Richter et al.*, 1992; *Oltmans et al.*, 1972; *Liauta-Teglivets et al.*, 1995; *van Bastelaere et al.*, 1995). The same appears to be true in *Haemophilus influenzae, Photobacterium leiognathi, Photobacterium phosphoreum, Archaeoglobus fulgidus, Helicobacter pylori, Dehalospirillum multivorans, Methanococcus jannaschii, Methanobacterium thermoautotrophicum* on basis of DNA sequence information (*Fleischmann et al.*, 1995; *Lee et al.*, 1994; *Klenk et al.*, 1997; *Tomb et al.*, 1997; *Bult et al.*, 1996).

We have discovered that in plants GTP cyclohydrolase II and 3,4-dihydroxy-2-butanone 4-phosphate synthase are fused. This means that the bifunctional enzyme is likely to have compounded structural constraints which make it susceptible for highly specific inhibition and thus a promising target for screening for inhibitors. Such a protein, useful for screening purposes may have both functions combined as in plants or some bacteria; or only one of these functions as in fungi or other bacteria.

It is a first object of the present invention to provide a method for screening chemical test samples for the presence or absence of inhibition of GTP cyclohydrolase II activity for finding an inhibitor.

This first object is achieved by a method for screening for the presence or absence of inhibition of GTP cyclohydrolase II activity, comprising the following steps:

(a) preparing a first aqueous mixture containing a protein having a GTP cyclohydrolase II sequence and GTP,

(b) reacting said first mixture during a predetermined period of time at a predetermined temperature and subsequently detecting the level of 2,5-diamino-6-ribosylamino-4(3H)-pyrimidinone 5'-phosphate,

(c) preparing a second aqueous mixture by including in said first mixture a predetermined amount of a chemical test sample

(d) reacting said second mixture during the predetermined period of time at the predetermined temperature and subsequently detecting the level of 2,5-diamino-6-ribosylamino-4(3H)-pyrimidinone 5'-phosphate,

(e) determining the presence of inhibition of GTP cyclohydrolase II by observation of whether the level detected in step (d) is lower than the level detected in step (b).

It is a second object of the invention to provide a method for screening for the presence or absence of resistance to an inhibition of a GTP cyclohydrolase II activity whereby the test sample contains a protein having a mutated plant-type GTP cyclohydrolase II sequence for finding a mutated enzyme that is resistant to a specific inhibitor.

The second object is achieved by a method for screening for the presence or absence of inhibition of GTP cyclohydrolase II comprising the following steps:

(a) preparing a first aqueous mixture containing a protein having a mutated plant-type GTP cyclohydrolase II sequence and GTP,

(b) reacting said first mixture during a predetermined period of time at a predetermined temperature and subsequently detecting the level of 2,5-diamino-6-ribosylamino-4(3H)-pyrimidinone 5'-phosphate,

(c) preparing a second aqueous mixture by including in said first mixture a predetermined amount of a specific inhibitor of said GTP cyclohydrolase II,

(d) reacting said second mixture during the predetermined period of time at the predetermined temperature and subsequently detecting the level of 2,5-diamino-6-ribosylamino-4(3H)-pyrimidinone 5'-phosphate,

(e) determining the presence of inhibition or resistance to inhibition respectively, of GTP cyclohydrolase II by observation of whether the level detected in step (d) is similar to the level detected in step (b).

It is a third object of the present invention to provide a method for screening chemical test samples for the presence or absence of inhibition of 3,4-dihydroxy-2-butanone 4-phosphate synthase activity for finding an inhibitor.

The third object is achieved by a method for screening for the presence or absence of inhibition of 3,4-dihydroxy-2-butanone 4-phosphate synthase activity, comprising the following steps:

(a) preparing a first aqueous mixture containing a protein having a 3,4-dihydroxy-2-butanone 4-phophate synthase sequence and ribulose 5-phosphate,

(b) reacting said mixture during a predetermined period of time at a predetermined temperature and subsequently detecting the level of 3,4-dihydroxy-2-butanone 4-phophate,

(c) preparing a second aqueous mixture by including in said first mixture a predetermined amount of a specific inhibitor for said 3,4-dihydroxy-2-butanone 4-phophate synthase,

(d) reacting said second mixture during the predetermined period of time at the predetermined temperature and subsequently detecting again the level of 3,4-dihydroxy-2-butanone 4-phophate,

(e) determining the presence of inhibition of 3,4-dihydroxy-2-butanone 4-phophate synthase by observation of whether the level detected in step (d) is lower than the level detected in step (b).

It is a fourth object of the invention to provide a method for screening test samples for the presence or absence of inhibition of 3,4-dihydroxy-2-butanone 4-phosphate synthase activity, whereby the test sample contains a protein having a mutated plant-type 3,4-dihydroxy-2-butanone 4-phosphate synthase sequence, for finding a mutated enzyme that is resistant to a specific inhibitor.

The fourth object is achieved by a method for screening for the presence or

absence of inhibition of 3,4-dihydroxy-2-butanone 4-phosphate synthase activity, comprising the following steps:

(a) preparing a first aqueous mixture containing
a protein having a mutated plant-type 3,4-dihydroxy-2-butanone 4-phosphate synthase sequence and ribulose 5-phosphate,

(b) reacting said mixture during a predetermined period of time at a predetermined temperature and subsequently detecting the level of 3,4-dihydroxy-2-butanone 4-phosphate,

(c) preparing a second aqueous mixture by including in said first mixture a predetermined amount of a specific inhibitor for said 3,4-dihydroxy-2-butanone 4-phosphate synthase,

(d) reacting said second mixture during the predetermined period of time at the predetermined temperature and subsequently detecting again the level of 3,4-dihydroxy-2-butanone 4-phosphate,

(e) determining the presence of resistance to inhibition of 3,4-dihydroxy-2-butanone 4-phosphate synthase by observation of whether the level detected in step (d) is similar to the level detected in step (b).

The enzyme sequence may be a plant type for finding herbicides, a fungal type for finding fungicides or a bacterial typ for finding inhibitors against pathogenic bacteria.

For performing the screening methods, kits of parts may be used, whereby the required components are partitioned such that upon mixing of the parts the enzymatic reaction can be initiated at a certain point in time.

Preferably the enzymes are promoted with a bivalent metal ion. In this case the enzymatic reaction may be stopped at a certain point in time by adding a chelating agent for said metal ion.

The level of the products of the reaction of the target enzymes may be determined

directly or by chemical or enzymatic derivatization.

The level of the product may either be determined at one predetermined point of time or consecutively at several predetermined points of time.

The invention will now be described in detail.

Sequence determination of plant enzymes

The known amino acid sequence of the RibA protein of *B. subtilis* (*Richter et al.*, 1993) was used to perform a sequence analysis with *Arabidopsis thaliana* EST-clone 41G4T7 available from the Arabidopsis Biological Resource Center, Ohio State University, USA, and a significant similarity was found with EST clone 41G4T7. It was determined that this sequence similarity concerned the 3,4-dihydroxy-2-butanone 4-phosphate synthase domain of the RibA protein of *B. subtilis*. This was surprising since EST 41G4T7 had been previously annotated as riboflavin synthase (Newman *et al.*, 1994).

The entire insert of EST clone 41G4T7 was sequenced by the automated dideoxynucleotide method using a primer walk strategy. The insert (designated i41G4T7) had a length of 2525 bp. The segment comprising bp 109-1297 is similar to the *ribA* gene of *B. subtilis*; the segments containing bp 563-1485 and 2262-2505 are identical with the *A. thaliana* sequence D45165 (Genbank/DDJB/EMBL/GSDB/NCBI accession no.) which was thought to be a GTP cyclohydrolase II (Kobayashi *et al.*, 1995). The transposon IS1 was located at BP 1486-2261.

Sequence comparison with the *B. subtilis* gene suggested that the EST clone 41G4T7 did not contain the entire 5' part of the gene under study. We therefore performed a Northern blot with mRNA from *Arabidopsis thaliana* var. Columbia using a hybridization probe obtained by PCR amplification of bp 39-1484 from

clone 41G4T7. This probe contained parts of the putative 3,4-dihydroxy-2-butanone 4-phosphate synthase domain as well as the putative GTP cyclohydrolase II domain. We found a single band of about 2300 bp.

In order to extend the known sequence in the 5' direction, a RACE experiment was performed by reverse PCR using mRNA from *A. thaliana* as template. The RACE product, which had a length of 800 bp, was sequenced and was shown to contain 556 bp which were not present in clone 41G4T7. The coding region of the extended cDNA contains 1629 bp specifying a predicted protein with 543 aa and a mass of 59,055 Da. The cognate gene is designated *ribA*. The sequence is shown in Annex B. The 5' untranslated region is 284 bp long. The 3' untranslated region has a length of 372 bp.

The putative 3,4-dihydroxy-2-butanone 4-phosphate synthase domain of the *Arabidopsis* gene is preceded by a sequence of about 120 amino acids which is devoid of similarity to any sequence in the database. The first 25 amino acids of the protein contain 13 serine residues (including a cluster of 4 consecutive serine residues). Thirtyfour residues in the N-terminal 120 amino acid residues are serine or threonine.

The chromosomal *ribA* gene of *Arabidopsis* was amplified in two parts by PCR using chromosomal DNA as template. The amplificates were sequenced. It was found that this gene (EMBL database accession no. AJ000053) includes six introns with a total of 700 bp.

Complementation experiments

The coding region of the *A. thaliana* gene was inserted into the expression vector pNCO113 (*Stüber et al.*, 1990) yielding the plasmid pAE. This plasmid was electrotransformed into *ribA* and *ribB* mutant strains of *E. coli* which are deficient of 3,4-dihydroxy-2-butanone 4-phosphate synthase and GTP cyclohydrolase II,

respectively, and which are, therefore, unable to grow on LB medium without supplementation of riboflavin (Katzenmeier, 1991). The plasmid pNCO113 transformed into the mutants served as a negative control. The recombinant mutant strains carrying the plasmid with the *ribA* gene of *Arabidopsis* grow at normal rate in the absence of external riboflavin. It follows that the *ribA* gene of *Arabidopsis* directs the synthesis of a protein which can serve as GTP cyclohydrolase II and as 3,4-dihydroxy-2-butanone 4-phosphate synthase in *E. coli* cells. The *A. thaliana* gene therefore codes for a bifunctional GTP cyclohydrolase II/ 3,4-dihydroxy-2-butanone 4-phosphate synthase.

Cloning of the ribA gene of tomato

A gene with similartiy to the *ribA* gene of *A. thaliana* was amplified from a tomato cDNA library by PCR using degenerate primers. The DNA segment was sequenced using a primer walk strategy and was shown to contain an open reading frame (EMBL database accession no. AJ002298) of almost the same size as the *A. thaliana* gene (Annex C). The predicted protein sequence contains 552 amino acids and has a calculated mass of 59793 Da. The predicted protein is similar to the RibA protein of *A. thaliana*. Most notably, it also has a serine and threonine rich N-terminus of about 120 amino acids preceding the 3,4-dihydroxy-2-butanone 4-phosphate synthase domain. Apart from the high serine/threonine content, the N-terminus of the tomato gene has little similarity to the *A. thaliana* N-terminus. The following 3,4-dihydroxy-2-butanone 4-phosphate synthase and cyclohydrolase II domains are very similar to the *Arabidopsis* enzyme.

By the method here described the corresponding gene and protein sequence of any other organism can be determined.

The deduced amino acid sequence of the *ribA* gene of *A. thaliana* shows also similiarity with bifunctional GTP cyclohydrolase II/3,4-dihydroxy-2-butanone 4-phosphate synthase sequences from *Actinobacillus pleuropneumoniae* and with

putative bifunctional enzymes from *Bacillus amyloliquefaciens*, *M. tuberculosis* and *Synechocystis sp*. The *A. thaliana* gene is also similar to genes from *Photobacterium phosphoreum* and *Photobacterium leiognathi*. The N-terminal 3,4-dihydroxy-2-butanone 4-phosphate synthase domain of the *A. thaliana* enzyme shows similarity to monofunctional 3,4-dihydroxy-2-butanone 4-phosphate synthase sequences not only from *Escherichia coli*, but also *H. influenzae* and *S. cerevisiae*. The C-terminal GTP cyclohydrolase II domain shows similarity to monofunctional GTP cyclohydrolase II sequences from not only *E. coli*, but also *H. influenzae*, *S. cerevisiae*, *Candida guilliermondii*, *P. phosphoreum* and *Azospirillum brasilense*.

Expression and purification of GTP cyclohydrolase II/3,4-dihydroxy-2-butanone 4-phosphate synthase from plants

Originator for the construction of an expression vector is cDNA of a plant, preferably of a dicotyle or monocotyle plant, for example cDNA from *Arabidopsis thaliana* or *Lycopersicon esculentum*. The *ribA* gene is amplified by PCR with specific primers and cDNA from the corresponding plant as template. In a variant process the gene may be amplified in two overlapping parts. The two parts are digested with a restriction enzyme in the overlapping area and ligated together afterwards to yield the complete gene. Alternatively the cDNA of one or both parts may originate from an existing EST-clone. If only the GTP cyclohydrolase II part or the 3,4-dihydroxy-2-butanone 4-phosphate synthase part of *ribA* should be expressed, only the corresponding part of the gene is amplified with the above described methods.

The RibA protein from various plants for example *A. thaliana* or *L. esculentum* includes a signal sequence of about 120 amino acids which was found not to be essential for enzyme activity. This signal sequence may be excluded in the recombinant DNA construct.

The amplified DNA fragment is modified by two consecutive PCR amplifications with modifying primers. In the first PCR reaction a ribosomal binding site preceding the start codon at an optimal distance is introduced at the 5'-end. A recognition site for a restriction enzyme, for example SalI, BspMI or Pvu1 is introduced at the 3'-end. The preferred recognition site is SalI. In the second PCR reaction the product of the first PCR reaction is used as template. At the 5'-end a recognition site for the restriction enzyme EcoRI preceding the ribosomal binding site is introduced with a modifying primer. The amplified DNA fragment is digested with the corresponding restriction enzymes, for example EcoRI and SalI. This DNA fragment is inserted into a vector DNA capable of autonomous replication in the host microorganism to give a recombinant plasmid containing said DNA (Fig. 2). This recombinant plasmid is used to transform the host microorganism, for example *Escherichia coli* or *Bacillus subtilis*. The preferred host is *E. coli*. The expression of plant proteins may be poor in the host organism. To enhance the expression level and/or to simplify the purification of the protein, the recombinant plasmid may include a gene or a part of a gene without stop codon preceding the *ribA* gene or parts of it in the same reading frame. A preferred gene for this purpose is the *malE* gene from *E. coli*. The expression of such a recombinant DNA generates a fusion protein between the maltose binding protein (MBP) from *E. coli* and the plant RibA protein. Various constructs are shown in Fig. 3. A construct with a signal sequence S is shown in Fig. 3a, with a maltose binding sequence malE and a signal sequence S in Fig. 3b and with a maltose binding sequence malE in Fig. 3c.

The strains harbouring the expression vectors can be cultivated in conventional culture media at 15 to 40 °C. The preferred culture medium is Luria Bertani medium and the preferred temperature is 37 °C. The *E. coli* strains are induced with 0.2 to 5 mM isopropyl-β-D-thiogalactosid at an optical density from 0.5 to 0.8. The cells are incubated between 2 and 12 h, preferably 5 h. The cells are harvested by centrifugation and washed with saline. The cells are lysed with lysozyme and/or disrupted with a sonifier. The MBP-ribA fusion protein is purified

from crude extract by affinity chromatography with an amylose resin.

Screening for the presence or absence of inhibition of GTP cyclohydrolase II activity

GTP is converted to 2,5-diamino-6-ribosylamino-4(3H)-pyrimidinone 5'-phosphate by the action of GTP cyclohydrolase II. The enzyme can be promoted by a bivalent metal ion such as Ba^{2+}, Ca^{2+}, Sr^{2+}, Co^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+} or Zn^{2+}. The preferred ion is Mg^{2+}. The reaction mixture should preferably contain an antioxidative substance such as dithiothreitol (DTT), dithioerythritol (DTE), butylhydroxyanisole (BHA) or butylhydroxytoluene (BHT). The assay can be started by adding one of the needed substances to a mixture of the others. Preferably the enzyme is added to a solution of GTP, Mg^{2+}, DTT in a buffer at pH 6 to 9,5, preferably 8,5. The reaction can be incubated for 1 to 60 min at 10 to 40 °C. Preferable it is incubated for 20 min. at 37 °C. The assay can be stopped by denaturating the enzyme with trichloroacetic acid, acetone, sodium dodecyl sulfate or heating at temperatures between 60 to 100 °C. The assay can also be stopped by chelating the metal ion with complexing agent such as EDTA, iminodiacetic acid, 8-hydroxyquinolin, diphenylcarbazid, dithizon or glyoxal-bis-(2-hydroxyanil). The preferred complexing agent is EDTA.

The assay is carried out with otherwise identical mixtures with and without test sample of a possible inhibitor. The enzyme product 2,5-diamino-6-ribosylamino-4(3H)-pyrimidinone 5'-phosphate can be detected directly without derivatization, preferably photometrically, preferentially after purification by HPLC. The pyrimidinone can be identified by UV absorbance at 293 nm. The extinction coefficient is 12,100 $M^{-1}cm^{-1}$. The product can also be monitored by diode array multiwavelength detection (200 - 600 nm). The assay may also be performed in a quartz cuvette without stopping the reaction. The reaction rate can then be determined directly by monitoring the absorbance of GTP at 252 nm and of 2,5-diamino-6-ribosylamino-4(3H)-pyrimidinone 5'-phosphate at 293 nm.

The pyrimidinone can also be detected by chemical derivatization, preferably by reaction with a vicinal dioxo compound (Fig. 4). Preferable are vicinal diketo compounds of the formula R^1-CO-CO-R^2, wherein R^1 and R^2 are independently aliphatic, alicyclic, aromatic or heteroaromatic residues, such as diphenyldiketone, phenyl-methyl-diketone, camphoro quinone; preferably C_{1-6} alkyl rests. The most preferred diketone is diacetyl (2,3-butanedione) (8). The reaction with 2,5-diamino-6-ribosylamino-4(3H)-pyrimidinone 5'-phosphate yields 6,7-dimethylpterin (9), which can be detected fluorometrically at an absorbance wavelength at 365 nm and an emission wavelength at 435 nm.

The enzyme activity of GTP cyclohydrolase II can also be monitored after enzymatic derivatization, preferably by converting 2,5-diamino-6-ribosylamino-4(3H)-pyrimidinone 5'-phosphate into 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione 5'-phosphate (10) by action of a fungal, preferably yeast, 2,5-diamino-6-ribosylamino-4(3H)-pyrimidinone 5'-phosphate reductase (Fig. 4); for the sequence Annex D is pointed out. This reductase can be expressed in *E. coli* with additional N- or C-terminal histidines by standard molecular biological techniques with DNA from *Saccharomyces cerevisiae* as template. Before used in the assay the reductase has to be purified for example on a metal chelate affinity column. The conversion needs NADPH or NADH as cosubstrate. The amount of 2,5-diamino-6-ribosylamino-4(3H)-pyrimidinone 5'-phosphate can be calculated by the consumption of NADH which can be monitored photometrically at 340 nm.

The screening can be done with a bifunctional enzyme of a monocotyledonous or dicotyledonous plant. It is also possible to use only the GTP cyclohydrolase II domain in a first step screening and bifunctional enzyme in a second step screening. It is also possible to use a bacterial or fungal enzyme.

Screening for the presence or absence of inhibition of 3,4-dihydroxy-2-butanone 4-phosphate synthase activity

Ribulose-5-phosphate is converted to 3,4-dihydroxy-2-butanone 4-phosphate by the action of 3,4-dihydroxy-2-butanone 4-phosphate synthase. The enzyme requires a bivalent metal ion such as Ba^{2+}, Ca^{2+}, Sr^{2+}, Co^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+} or Zn^{2+}. The preferred ion is Mg^{2+}. The assay can be started by adding one of the needed substances to a mixture of the others. Preferably the enzyme is added to a solution of ribulose-5-phosphate and Mg^{2+} in buffer at pH 6 to 9,5, preferably 7,5. The assay is carried out with otherwise identical mixtures with and without a test sample of a possible inhibitor. The reaction can be incubated for 1 to 90 min at 10 to 40 °C. Preferably it is incubated for 60 min. at 37 °C. The substrate for 3,4-dihydroxy-2-butanone 4-phosphate synthase, ribulose 5-phosphate is commercially available, but at considerable cost. It is preferably generated *in situ* by isomerization of ribose 5-phosphate catalyzed by pentose phosphate isomerase. In this case a mixture containing preferably pentose phosphate isomerase, ribose-5-phosphate and Mg^{2+} in buffer at pH 6,0 to 9,5 incubated for 1 to 20 min before adding 3,4-dihydroxy-2-butanone 4-phosphate synthase and optionally the test sample. The assay can be stopped by chelating the metal ion with complexing agent such as EDTA, iminodiacetic acid, 8-hydroxyquinolin, diphenylcarbazid, dithizon or glyoxal-2-bis-(2-hydroxyanil). The preferred complexing agent is EDTA. For detection enzymatic derivatization is possible. Preferably, the product 3,4-dihydroxy-2-butanone 4-phosphate can be converted enzymatically with 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione to 6,7-dimethyl-8-ribityllumazine in the presence of 6,7-dimethyl-8-ribityllumazine synthase or to riboflavin in the presence of 6,7-dimethyl-8-ribityl-lumazine synthase and riboflavin synthase. 5-Amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione decomposes rapidly in the presence of molecular oxygen and has to be stored, therefore, as aqueous solution containing an antioxidative substance such as dithiothreitol (DTT) or dithioerythrol (DTE) at temperatures below 0 °C. The assay can be stopped and the detection reaction started by adding a solution of complexing agent, 5-amino-6-ribitylamino-

2,4(1H,3H)-pyrimidinedione, antioxidative substance, 6,7-dimethyl-8-ribityllumazine synthase and optionally riboflavin synthase (Fig. 5). After incubation for 10 to 60 min at 20 to 40 °C the detection reaction can be stopped by denaturating the enzymes with trichloroacetic acid, acetone, sodium dodecyl sulfate or heating at temperatures between 60 to 100 °C. 6,7-Dimethyl-8-ribityllumazine can be detected photometrically at 410 nm, if only the lumazine synthase is added. Riboflavin can be detected photometrically at 445 nm, if the lumazine synthase and the riboflavin synthase are added. The extinction coefficient for riboflavin (pH 1, 445 nm) is 11,500 $M^{-1}cm^{-1}$. The extinction coefficient for 6,7-dimethyl-8-ribityllumazine (pH 1, 410 nm) is 10,300 $M^{-1}cm^{-1}$. There are two molecules of 3,4-dihydroxy-2-butanone 4-phosphate required for the formation of one molecule riboflavin.

3,4-Dihydroxy-2-butanone 4-phosphate can also be detected after chemical derivatization, preferably with an aromatic or heteroaromatic ortho-diamine (Fig. 5). The aromatic or heteroaromatic ring may be substituted or unsubstituted. It may contain 1 to 3 substituents selected for the group of alkyl, halogen, alkoxy.

It is possible to use the bifunctional enzyme of a monocotyledonous or dicotyledonous plant. It is also possible to use only the 3,4-dihydroxy-2-butanone 4-phosphate synthase domain in a first step screening and bifunctional enzyme in a second step screening. It is also possible to use a bacterial or fungal enzyme.

The screening methods described above may readily be used for screening for inhibition resistance after corresponding modification. It is merely necessary to add a specific inhibitor (previously determined by screening) and to use a biological test sample which contains a mutant of the plant-type enzyme in question.

The invention will now be described by specific Examples.

Reference Example 1

Isolation of the A. thaliana ribA gene

1 g of 2 weeks old *Arabidopsis thaliana* var. Columbia plants (stems and leafs) were frozen and homogenised in liquid nitrogen. 8 ml of 50 mM trisodium citrate containing 600 g guanidine thiocyanate, 5 g sodium-N-lauroylsarcosine, and 5 ml 2-mercaptoethanol per liter were added. This mixture was added carefullly to 3 ml of a sterile solution containing 959 g CsCl and 37.2 g EDTA per liter. The mixture was centrifuged at 33,000 rpm at 18°C for 24 h. The supernatant was discarded and the pellet was airdried for 10 min. The pellet was dissolved in 360 µl H_2O (bidestillated, sterile). The solution was centrifuged at 14,000 rpm for 10 min. The supernatant was mixed with 40 µl of 3 M sodium acetate and 1 ml of ethanol. The RNA was precipitated overnight at -20°C, centrifuged at 14,000 rpm at 4°C for 15 min and washed twice with 500 µl of 75 % ethanol. The pellet was airdried and dissolved in 500 µl of H_2O (bidestilled, sterile). mRNA from this crude RNA fraction was isolated with the Oligotex mRNA-Isolation kit (Quiagen). The RNA solution (500 µl) was mixed with 500 µl 20 mM Tris hydrochloride, pH 7.5 containing 1 M NaCl, 2 mM EDTA and 0.2 % SDS. 30 µl Oligotex suspension were added and the mixture was incubated for 3 min at 65° and for 10 min at room temperature. The suspension was centrifuged for 2 min at 14,000 rpm and the supernatant was aspirated. The pellet was washed twice with 1 ml of 10 mM Tris hydrochloride, pH 7.5 containing 150 mM NaCl and 1 mM EDTA. The mRNA was eluted twice with 50 µl of preheated (70°C) 5 mM Tris hydrochloride buffer, pH 7.5

A. thaliana mRNA (4 µg) was electrophoresed at 40 V on a 2.2 M formaldehyde 1 % agarose gel. 4 µl of a RNA ladder (Gibco) was electrophoresed as a reference. The reference lane was cut off and stained in a 0.1 % toluidine blue solution for 10 min The other part of the gel was washed 4 times with H_2O (DEPC treated) and transferred overnight to a Nytrans nylon membrane with 0.3 M trisodium citrate, pH 7.0 containing 3 M NaCl using the turboblotter system from Schleicher and Schuell.

The probe was amplified from plasmid 41G4T7 by PCR. The plasmid was isolated from 5 ml of fresh overnight culture of EST-clone 41G4T7 using the mini plasmid isolation kit from Quiagen. The bacterial pellet was resuspended in 0.3 ml of 50 mM Tris hydrochloride, pH 8.0 containing 10 mM EDTA and 100 µg/ml RNase. 0.3 ml of 200 mM sodium hydroxide containing 1 % SDS were added and incubated 5 min at room temperature. 0.3 ml of chilled 3.0 M sodium acetate pH 5.5 were added and incubated on ice for 10 min. The mixture was centrifuged for 15 min at 14,000 rpm in a minifuge. The supernatant was removed and applied onto a Quiagen-tip 20 which was previously equilibrated with 1 ml of 50 mM MOPS, pH 7.0 containing 750 mM NaCl, 15 % ethanol and 0.15 % Triton X-100. The Quiagen-tip was washed 4 times with 1 ml of 50 mM MOPS, pH 7.0 containing 1000 mM NaCl and 15 % ethanol. The DNA was eluted with 0.8 ml of 50 mM Tris hydrochloride, pH 8.5 containing 1250 mM NaCl and 15 % ethanol. The DNA was precipitated with 0.7 volumes of isopropanol, centrifuged at 14,000 rpm for 30 min and washed with 1 ml of cold 70 % ethanol. The DNA sequence of 41G4T7 was previously determined with a primer walk strategy. Sequencing was performed by the automated dideoxynucleotide method using an ABI Prism 377 DNA sequencer from Applied Biosystems Inc. with the ABI Prism Sequencing Analysis Software. EST-clone 41G4T7 was obtained from the Arabidopsis Biological Resource Center, Ohio State University, USA. The PCR mixture contained 25 pmol primer CTCCTCCTGCACCAGCCAATGG, 25 pmol primer TCAAGTTTCTCAGACAG ATCAAATG, 2U of Taq polymerase, 10 µl of buffer (Primezyme, Biometra), 1.6 ng of plasmid 41G4T7, and 20 nmol dNTPs in a total volume of 100 µl H_2O.

The mixture was denaturated at 94°C for 5 min. Then 25 cycles (30 sec at 94°C, 45 sec at 50°C, 90 sec at 72°C) were performed. After incubation for 7 min at 72°C, the mixture was cooled at 4°C and the DNA was purified with a PCR purification kit (Quiagen). 5 Volumes of buffer PB (Quiagen) were added to 1 volume of the PCR reaction, applied to a Quiaquick column and centrifuged for 1 min at 14,000 rpm. The flow through was discarded. 0.75 ml buffer PE

(Quiagen) were added to the column and centrifuged as before. The flow through was discarded and the column was centrifuged for an additional 1 min at 14,000 rpm. The column was placed in a clean 1.5 ml eppendorf tube. 50 µl of H_2O (bidestilled, sterile) were added to the column and it was centrifuged for 1 min at 14,000 rpm. The flow through contains the purified DNA. 105 ng of the purified DNA in 15 µl H_2O were heated at 100°C for 5 min. After cooling on ice, 5 µl of buffer containing random hexamer primers, dATP, dGTP, dTTP, 5 µl of ^{32}P-dCTP (50µCi) and 0.8 µl of Klenow polymerase (4 U) were added. The mixture was incubated for 1 h. The probe DNA (bp 39-1484 from EST sequence 41G4T7) was precipitated two times with 2 M NH_4SO_4. The pellet was dissolved in 200 µl of H_2O.

The membrane was prehybridized in a mixture of 50 % formamide, 50 % SSPE/Denhards solution/SDS (1.0 g Ficoll, 1.0 g polyvinylpyrrolidone, 1.0 g BSA, 87.6 g NaCl, 13.8 g NaH_2PO_4, 3.7 g EDTA, 10 g SDS per liter) for 1 h at 42°C. 200 µl probe were added, and hybridization was performed at 42°C in 50 % formamide, 50 % SSPE/Denhards solution/SDS for 12 h. The membranes were then washed twice with 2xSSPE containing 0.1 % SDS at room temperature for 15 min. The radioactive bands were detected for 3.5 h on a PhotoImager (Molecular Dynamics). A single band of about 2300 bp was found.

A 5' RACE was performed with the 5' RACE system from GibcoBRL. The cDNA was generated with a specific primer from *A. thaliana* RNA. The mixture contained 2.5 pmol of primer TCAACAGATGCTTCAGTGTGTCC and 990 ng of *A. thaliana* RNA in a total volume of 15 µl. The mixture was denaturated at 70°C for 10 min and cooled on ice. 2.5 µl of 10x reaction buffer (Gibco), 3 µl 25 mM $MgCl_2$, 1 µl 10 mM dNTPs and 2.5 µl 0.1 M dithiothreitol were added. The mixture was incubated at 42°C for 2 min, 1 µl (200 U/µl) of reverse transcriptase SuperscriptII (Gibco) were added. The mixture was incubated at 65°C for another 15 min. It was then centrifuged, the temperature was adjusted to 55°C, and 1 µl of RNAseH (2 U/µl) was added. After another 10 min

incubation at 55°C the mixture was cooled on ice.

Subsequently the cDNA was amplified using an anchor primer (GibcoBRL) and a specific nested primer (CCTTCATTTTCCCTATCTTCATCATC). The product was purified by electrophoresis in a 2 % agarose gel. The band at 800 bp was extracted using a gel extraction kit (Quiagen) and sequenced. The DNA fragment was excised from the agarose gel with a scalpel. 3 volumes of buffer QX1 (Quiagen) were added to 1 volume of the excised gel and incubated at 50°C for 10 min. One gel volume of isopropanol was added. To bind DNA, the sample was applied to a Quiaquick column and centrifuged for 1 min at 14,000 rpm. The flow through was discarded. 0.75 ml buffer PE (Quiagen) were added to the column and centrifuged as before. The flow through was discarded and the column was centrifuged for an additional 1 min at 14,000 rpm. The column was placed in a clean 1.5 ml eppendorf tube. 50 μl of H_2O (bidestilled. sterile) were added to the column and it was centrifuged for 1 min at 14,000 rpm. The flow through contained 4.2 μg of the purified DNA.

Reference Example 2

Construction of an expression clone

Using plasmid from EST clone 41G4T7 as template, the cDNA insert was amplified from bp -75 to 1485 by PCR. The reaction mixtures contained 10 pmol of primer TCAAGTTTCTCAGACAGATCAAATG, 10 pmol primer GAAACAGCT ATGACCATGATTACG, 4.5 ng of plasmid 41G4T7, 2U of Taq polymerase, 10 μl of buffer (Primezyme, Biometra), and 20 nmol of dNTPs in a total volume of 100 μl.

The mixture was denaturated at 94°C for 5 min. 25 PCR cycles (60 sec at 94°C, 60 sec at 50°C, 120 sec at 72°C) were performed. After another 7 min incubation at 72°C, the mixture was cooled at 4°C, and the DNA was purified with a PCR purification kit (Quiagen). The PCR product and the RACE product

from (Reference Example 1) were digested with restriction enzyme BsgI (New England Biolabs). 5 µl New England Biolabs buffer 4, 1 µl S-adenosylmethionin (4 mM), 5 µl BsgI (7.5 U), 40 µl PCR product (1.6 µg) resp. 40 µl RACE product (3.2 µg).

Both fragments were incubated at 37°C for 2 h, purified as before and ligated together with T4-ligase, 4 µl of 5x buffer (Gibco), 1 µl of T4-ligase (4U), 2 µl of PCR product (60 fmol), 0.5 µl of RACE product (65 fmol), and 12.5 µl of H_2O.

The mixture was incubated at 4°C for 12 h. The completed gene was amplified by PCR with specific terminal primers. At the 5' end a recognition site for the restriction enzyme EcoRI preceding a ribosomal binding site at an optimal distance to the start codon was introduced by PCR with modifying primers. At the 3' end a recognition site for the restriction enzyme SalI was introduced after the stop codon by PCR with modifying primer.

First PCR
10 pmol primer GAGGAGAAATTAACTATGTCTTCCATCAATTTATCCTC, 10 pmol primer ACGCGTCGACGGTTCGTCCTGGTTTTTAAGC, 2U of Taq polymerase, 10 µl of buffer (Primezyme, Biometra), 1 µl of the ligation mixture, and 20 nmol of dNTPs in a total volume of 100 µl.

The mixture was denaturated at 94°C for 5 min. Then 25 cycles (60 sec at 94°C, 60 sec at 50°C, 120 sec at 72°C) were performed. After another 7 min incubation at 72°C, the mixture was cooled at 4°C and the DNA was electrophoresed on a 0.8 % agarose gel. The band at 1650 bp was purified with a gel extraction kit (Quiagen).

Second PCR (Two identical PCRs with 100 µl each were performed to obtain a higher yield)

10 pmol primer CAATTTGAATTCATTAAAGAGGAGAAATTAACTATG, 10 pmol primer ACGCGTCGACGGTTCGTCCTGGTTTTTAAGC, 2U of Taq polymerase, 10 µl of buffer (Primezyme, Biometra), 4 µl of purified PCR1 product, and 20 nmol dNTPs in a total volume of 100 µl.

The mixture was denaturated at 94°C for 5 min. Then 25 cycles (60 sec at 94°C, 60 sec at 50°C, 120 sec at 72°C) were performed. After another 7 min incubation at 72°C the mixture was cooled at 4°C and the DNA was purified with a PCR purification kit (Quiagen). Plasmid pNCO113 (Stüber *et al.*, 1990) was isolated as described for plasmid 41G4T7 in (Reference Example 1).

The PCR product and the plasmid pNCO 113 were digested with the restriction enzyme SalI (10 µl SalI buffer (NEB), 1 µl of BSA 100 µg/ml, 40 U of SalI (NEB), 2.4 µg of PCR2 product resp. 4.2 µg of pNCO113 in a total of 100 µl) at 37°C for 3 h, purified with a PCR purification kit (Quiagen) and digested with the restriction enzyme EcoRI (20 µl of OPA buffer (Phamacia), 48 µl of SalI digested PCR2 product resp. 48 µl of SalI digested pNCO113, 2 µl (20 U/µl) of EcoRI (Phamacia) and 30 µl of H_20) at 37°C for another 2 h and purified with a PCR purification kit. The digested PCR2 product and plasmid pNCO113 were ligated together with T4-ligase, yielding plasmid pAE: 44 fmol of pNCO113, 111 fmol of PCR2 product, 4 µl of buffer (Gibco), and 1 µl of T4 ligase (1 U) in a total of 20 µl.

The mixture was incubated over night at 4°C, purified with a PCR purification kit and transformed into electrocompetent *E. coli* XL I cells by electroporation.

Preparation of the electrocompetent cells: One liter of Luria-Bertani-media was inoculated with 1/100 volume of fresh overnight culture. The cells were grown at 37 °C with vigorous shaking to an optical density of 0.5 to 0.7. The cells were chilled on ice for 20 min and centrifuged in a cold rotor at 4,000 g for 15 min at 4 °C. The supernatant was removed and the pellet resuspended in 1 liter

of ice-cold sterile 10 % glycerol. The cells were centrifuged two times as described before and resuspended the first time in 0.5 liter and the second time in 20 ml of ice-cold sterile 10 % glycerol. The cells were centrifuged an additional time and the pellet was resuspended to a final volume of 2 to 3 ml in ice-cold 10 % glycerol. This suspension was frozen in aliquots of 80 µl and stored in liquid nitrogen.

Electro-transformation using the Gene Pulser apparatus from Biorad: The electrocompetent cells were thawn at room temperature and placed on ice. 40 µl of the cell suspension were mixed with 1 µl of the ligation mixture and transferred into a sterile 0.2 cm cuvette (Biorad). The suspension was shaked to the bottom and the cuvette was placed into the chamber slide. The chamber slide was pushed into the chamber and a pulse was applied (2.50 kV, 25 µF, Pulse Controller 200 Ω). The cuvette was removed from the chamber and the cells were suspended in 1 ml of SOC medium (2 % casein hydrolysate, 0.5 % yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose). The suspension was shaked 1 h at 37°C and plated on LB media supplemented with 150 mg ampicillin per liter. Plasmids from different clones were isolated (pAE 1 -pAE11) as described in Reference Example 1 for 41G4T7.

Reference Example 3

Construction of a malE-ribA fusion clone

The ribA gene was amplified by PCR using plasmid pAE11 (from *A. thaliana* ribA expression clone) as template. Plasmid pAE11 was isolated as described for plasmid 41G4T7 in (Reference Example 1). A recognition site for the restriction enzyme EcoRI preceding the start codon was introduced at the 5' end with a modifying primer.

PCR mixture: 10 pmol primer GTTCAGAATTCATGTCTTCCATCAATTT ATCCTC, 10 pmol primer ACGCGTCGACGGTTCGTCCTGGTTTTTAAGC, 2U

Taq polymerase, 10 µl buffer (Primezyme, Biometra), 1.7 ng plasmid pAE11, and 20 nmol dNTPs in a total volume of 100 µl.

The mixture was denaturated at 94 °C for 5 min. Then 25 cycles (60 sec at 94 °C, 60 sec at 50 °C, 120 sec at 72°C) were performed. After another 7 min incubation at 72°C the mixture was cooled at 4°C and the DNA was purified with a PCR purification kit (Quiagen).

The PCR product and the plasmid pMal-c2 (New England Biolabs) were digested with the restriction enzyme SalI (10 µl SalI buffer (NEB), 1 µl BSA 100 µg/ml, 60 U SalI (NEB), 2.0 µg PCR product resp. 0.4 µg pMal-c2 in a total volume of 100 µl) at 37°C for 2 h, purified with a PCR purification kit (Quiagen) and digested with the restriction enzyme EcoRI (20 µl OPA buffer (Phamacia), 48 µl SalI digested PCR product resp. 48 µl SalI digested pMal-c2, 3 µl EcoRI (20 U/µl) (Phamacia) and 30 µl H_2O) at 37°C for another 2 h and purified with a PCR purification kit. The digested PCR product and plasmid pMal-c2 were ligated together with T4-ligase, yielding plasmid pMal_ribA: 9 fmol pMal-c2, 30 fmol PCR product, 4 µl buffer (Gibco), and 1 µl T4-ligase (1 U) in a total volume of 10 µl. The mixture was incubated over night at 4°C. This plasmid contains the *A. thaliana* ribA gene in the same reading frame as the malE gene, resulting in the expression of a MBP-RibA fusion protein after induction with IPTG. The plasmid pMal_ribA was transformed into *E. coli* XL I cells by electroporation as described in (Reference Example 2).

Reference Example 4

Construction of a malE-ribA fusion clone without transit peptide sequence

The ribA gene exclusive the first 384 bp coding for a putative signal sequence was amplified by PCR using plasmid pAE11 (from *A. thaliana* ribA expression clone) as template. A recognition site for the restriction enzyme EcoRI preceding serine128 was introduced at the 5' end with a modifying primer.

PCR mixture: 10 pmol primer GTTCAGAATTCTCTTCTATCCCCGAGGC, 10 pmol primer ACGCGTCGACGGTTCGTCCTGGTTTTTAAGC, 2 U Taq polymerase, 10 µl buffer (Primezyme, Biometra), 1.7 ng plasmid pAE11, and 20 nmol dNTPs in a total volume of 100 µl.

The mixture was denaturated at 94 °C for 5 min. Then 95 cycles (60 sec at 94°C, 60 sec at 50°C, 120 sec at 72°C) were performed. After another 7 min incubation at 72°C the mixture was cooled at 4°C and the DNA was purified with a PCR purification kit (Quiagen).

The further steps were analogous to the construction of pMal_ribA in Reference Example 3. The plasmid encoding a MBP-RibA fusion protein without the putative transit peptide sequence of RibA was named pMal_ribAS.

Preparation Example 1
Preparation and purification of the MBP-RibA fusion proteins

0.5 l Luria Bertani (LB) medium containing 75 mg ampicillin were inoculated with 20 ml overnight culture of *E. coli* strain XL I harboring plasmid pMal_ribA resp. pMal_RibAS. The culture was grown in shaking culture at 37 °C. At an optical density (600 nm) of 0.8 the culture was induced with 1 mMol IPTG. The culture was grown for another 5 h. The cells were harvested by centrifugation for 20 min at 5000 rpm and 4 °C. The cells were washed with 0.9 % NaCl solution, centrifugated as above and frozen at -20 °C for storage.

The cells were thawed in 10 ml 20 mM Tris hydrochloride pH 7.4 containing 200 mM NaCl, 1 mM EDTA, 6 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol and 1 mg lysozyme (buffer A). The mixture was incubated at 37°C for 1 h, cooled on ice and sonified 6 x 10 sec (Branson sonifier level 4). The suspension was centrifugated at 15,000 rpm at 4 °C for 20 min. The supernatant was diluted 1:5 with buffer A and applied to a 1 ml column of

amylose resin (New England Biolabs) previously equilibrated with 8 ml buffer A. The column was washed with 12 ml buffer A without lysozyme. The fusion protein was eluted from the column with 4 ml 20 mM Tris hydrochloride pH 7.4 containing 200 mM NaCl, 1 mM EDTA and 10 mM maltose. 9.8 mg MBP-ribAS and 5.6 mg MBP-ribA protein were obtained. The proteins were identified by SDS-PAGE. MBP-ribAS showed a band at 89 kDa and MBP-ribA showed a band at 102 kDa.

Preparation Example 2 "reductase"
Construction and purification of a 2,5-diamino-6-ribosylamino-4 (3H)-pyrimidinone-5-phosphate reductase expression clone.

A PCR was carried out containing 10 pmol primer CATGCCATGGGTTCTTTGAC ACCACTGTGTGAAG, 10 pmol primer TATTATGGATCCTTAGTCATCGGCCAG TCTCGC, 2 U of taq polymerase, 10 µl of buffer (Primezyme, Biometra), 20 nmol of dNTPs and 30 ng of *Saccharomyces cerevisiae* DNA in a total of 100 µl. The mixture was denaturated at 94°C for 5 min. Then 30 cycles (30 sec at 94°C, 30 sec at 50°C, 90 sec at 72°C) were performed. After another 7 min. incubation at 72°C the mixture was cooled at 4°C and the DNA was purified with a PCR purification kit (Quiagen). The PCR product and the plasmid pNCO-H6 were digested with the restriction enzymes NcoI and BamHI (20 µl OPA buffer (Pharmacia), 40 U BamHI, 40 U NcoI and 2.5 µg PCR product resp. 1 µg pNCO-H6 in a total of 100 µl) at 37°C for 3 h. The digested DNA was purified with a PCR purification kit (Quiagen) and ligated together with T4-ligase (40 fmol of pNCO-H6, 80 fmol of the PCR product, 4 µl of buffer (Gibco), 1 U T4-ligase in a total of 20 µl). The mixture was incubated overnight, purified with a PCR purification kit (Quiagen) and transformed in electrocompetent *E. coli* XL1 cells as described in Reference Example 1.

Plasmid pNCO-H6 is essentially identical to plasmid pNCO113 (see reference Example 2) but the DNA sequence between the NcoI and the BamHI recognition

site is substituted by the DNA sequence: CATGCACCACCACCACCACCACGC GTCCATGGCCGCGGATCC .

0.5 l Luria Bertani (LB) medium containing 75 mg ampicillin were inoculated with 20 ml overnight culture of *E. coli* cells harbouring the plasmid pNCO-H6 with the gene for 2,5-diamino-6-ribosylamino-4(3H)-pyrimidinone-5-phosphate reductase. The culture was grown in a shaking culture at 37°C. At an optical density (600nm) of 0.8 the culture was induced with 1 mMol IPTG. The cells were harvested by centrifugation for 20 min at 5000 rpm. The cells were washed with 0.9 % saline and centrifuged as above. The cells were suspended in 10 ml 50 mM phosphate buffer pH 7.5 containing 0.6 mM phenylmethylsulfonyl fluoride, cooled on ice and sonified 6 x 10 sec (Branson sonifier level 4). The suspension was centrifuged at 15000 rpm at 4 °C for 20 min. The supernatant contained about 80 mg protein and was loaded onto a HiTrap (Pharmacia) metal chelate affinity column (volume 5 ml), charged with Ni^{2+} ions. The column was washed with 50 ml of 50 mM phosphate buffer pH 7.5. The 2,5-diamino-6-ribosylamino-4(3H)-pyrimidinone-5-phosphate reductase was eluted with 10 ml of 50 mM phosphate buffer pH 7.5 containing 500 mM imidazole. The elute was analyzed on a SDS-PAGE. The desired 2,5-diamino-6-ribosylamino-4(3H)-pyrimidinone-5-phosphate reductase has a molecular weight of 27 kDa.

Screening Example 1
Screening of GTP cyclohydrolase II activity

Assay mixtures contained 100 mM Tris hydrochloride pH 8.5, 5 mM $MgCl_2$, 5 mM dithiothreitol, 1 mM GTP and 20 µl enzyme (80 µg) sample as given in table 1 in a total volume of 80 µl. Separately, to these mixtures 0.5 and 5.0 mM sodium pyrophosphate, 0.5 and 5.0 mM methylene-bisphosphonate was added. They were incubated at 37 °C for 30 min. A derivatization solution (20 µl) of 5 % diacetyl and 250 mM EDTA was added and each mixture incubated at 90 °C for 1 h. The diacetyl reacts with the enzyme product to yield 6,7-dimethylpterin,

which was subsequently measured by reverse-phase HPLC on a column of Nucleosil RP18. The eluent contained 100 mM ammonium formate and 25 % methanol. The effluent was monitored fluorometrically (excitation 365 nm; emission 435 nm). One unit of enzyme activity catalyzes the formation of 1 nmol of 2,5-diamino-6-ribosylamino-4(3H)-pyrimidinone 5'-phosphate per h at 37 °C.

Screening Example 2

Assay mixtures contained 100 mM Tris hydrochloride pH 8.5, 10 mM $MgCl_2$, 5 mM dithiothreitol, 0.5 mM GTP and 20 µl enzyme sample. Separately to these mixtures 0.25 and 2.5 mM 2,6-diamino-5-formamido-pyrimidinon or 0.25 and 2.5 mM1-hydroxy-ethylidene-1,1-bis-phosphoric acid were added, and the mixtures were incubated at 37°C for 30 min. The further steps were identical to Screening Example 1.

Table 1

Enzyme	Test compound (mM)	Activity
pMal_ribA maltose eluate	pyrophosphate (0)	1,9 U (100%)
pMal_ribA maltose eluate	pyrophosphate (0,5)	0,7 U (37%)
pMal_ribA maltose eluate	pyrophosphate (5,0)	0,1 U (5%)
pMal_ribAS maltose eluate	pyrophosphate (0)	2,3 U (100%)
pMal_ribAS maltose eluate	pyrophosphate (0,5)	0,5 U (22%)
pMal_ribAS maltose eluate	pyrophosphate (5,0)	0,05 U (2%)
pMal_ribA maltose eluate	methylenediphosphonic acid trisodium salt (0)	2,4 U (100%)
pMal_ribA maltose eluate	methylenediphosphonic acid trisodium salt (0,5)	3,1 U (130%)
pMal_ribA maltose eluate	methylenediphosphonic acid trisodium salt (5,0)	2,6 U (108%)
pMal_ribAS maltose eluate	methylenediphosphonic acid trisodium salt (0)	2,5 U (100%)
pMal-ribAS maltose eluate	methylenediphosphonic acid trisodium salt (0,5)	3,5 U (140%)
pMal_ribAS maltose eluate	methylenediphosphonic acid trisodium salt (5,0)	2,1 U (84%)

Screening Example 3

The screening Example 1 is repeated with the exception that the derivatization solution is not added. Instead the assay mixtures contains additionally 100 µg 2,5-diamino-6-ribosylamino-4(3H)-pyrimidine 5'-phosphate reductase and 0.1 mM NADH and the total volume is 500 µl. The assay mixture is incubated in a cuvette at 37 °C and the absorbence at 340 nm is monitored. This gives the rate of consumed NADH which is equivalent to the rate of formed 2,5-diamino-6-ribosylamino-4(3H)-pyrimidine 5'-phosphate.

Screening Example 4

Assay mixture contains 100 mM Tris hydrochloride, pH 8.5, 5 mM $MgCl_2$, 5 mM dithiothreitol, 1 mM GTP and 20 µl enzyme (80 µg MBP-ribA) sample in a total volume of 80 µl. Analogous mixtures contain additionally 0.5 and 5.0 mM sodium pyrophosphate. After incubation for 30 min. at 37°C the reaction is stopped by adding 20 µl of 50 mM EDTA. The enzyme product, 2,5-diamino-6-ribosylamino-4(3H)-pyrimidinedione 5'-phosphate is determined by HPLC with a reverse phase Nucleosil RP18 column. The eluent contains 0.6 % isopropanol and 1 % triethylammonium phosphate, pH 7.0. The product is identified by UV absorbance at 293 nm and quantified by integration. The extinction coefficient is 12,100 $M^{-1}cm^{-1}$.

Screening Example 5

Assay mixture contains 100 mM Tris hydrochloride, pH 8.5, 5 mM $MgCl_2$, 0.1 mM GTP and 20 µl enzyme (80 µg MBP-ribA) sample in a total volume of 500 µl. Analogous mixtures contain additionally 0.5 and 5.0 mM sodium pyrophosphate. The assay is performed in a quartz cuvette at 37°C under exclusion of oxygen. The reaction rate is determined directly by monitoring the absorbance of GTP at 252 nm and of 2,5-diamino-6-ribosylamino-4(3H)-pyrimidinedione 5'-phosphate at 293 nm.

Screening Example 6

A part C containing 0 resp. 2.0 resp. 20 mM sodium pyrophosphate in 20 µl H_2O is added to a mixture A containing 400 mM Tris hydrochloride pH 8.5, 30 mM $MgCl_2$, 20 mM DTT and 50 µg MBP_ribA enzyme in a total of 20 µl. A third solution B containing 4 mM GTP in 20 µl H_2O is added to start the reaction. The mixture is incubated at 35°C for 30 min. 20 µl of mixture E containing 5% diacetyl and 250 mM EDTA is added and heated for 1 h at 90°C. The amount of 6,7-dimethylpterin was determined fluorometrically (excitation 365 nm; emission 435 nm) by comparison with a 3 µM 6,7-dimethylpterin standard.

Screening Example 7

Screening of 3,4-dihydroxy-2-butanone 4-phoshate synthase activity.
Assay mixture contains 300 mM potassium phosphate pH 7,5, 20 mM $MgCl_2$, 10 mM ribose 5-phosphate and 0.1 U pentose-phosphate isomerase in a total volume of 30 µl. They were incubated at 37 °C for 15 min. and 10 µl of the enzyme sample and 10 µl of H_2O resp. 10 µl of 100 mM pyruvaldehydoxim as given in table 2 were added. The mixture (50 µl) was incubated for 1 h. A solution (50 µl) containing 200 mM potassium phosphate pH 7.5, 40 mM dithiothreitol, 50 mM EDTA, 4 mM 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione and 10 U of the lumazine synthase/riboflavin synthase complex from *Bacillus subtilis* was added. The lumazine synthase/riboflavin synthase is prepared as described by Schott *et al.*, 1990.The mixture was incubated 1 h at 37 °C and then denaturated at 95 °C for 5 min. Riboflavin was determined by reverse-phase HPLC on a column of Nucleosil RP18. The eluent contained 100 mM ammonium formate and 40 % methanol. The effluent was monitored fluorometrically (excitation 445 nm; emission 516 nm). One unit of enzyme activity catalyzes the formation of 1 nmol of 3,4-dihydroxy-2-butanone 4-phosphate per h at 37 °C.

Table 2

Enzyme	Conc. (Pyruvaldehydoxim)	Activity
pMal_ribAS maltose eluate	0 mM	27 U (100 %)
pMal_ribAS maltose eluate	20 mM	14 U (52 %)

Screening Example 8

The assay is performed as described in screening Example 7. Instead of the lumazine synthase/riboflavin synthase complex only 6,7-dimethyl-8-ribityl-lumazine synthase is added. 6,7-Dimethyl-8-ribityl-lumazine is determined by reverse phase HPLC on a column of nucleosil RP18. The eluent contains 10% methanol and 30 mM formic acid. The effluent is monitored fluorometrically (excitation 408 nm; emission 487 nm).

Screening Example 9

Assay mixture contains 300 mM potassium phosphate pH 7.5, 20 mM $MgCl_2$, 10 mM ribose 5-phosphate and 0.1 U pentose-phosphate isomerase in a total volume of 30 µl. They are incubated at 37°C for 15 min. 10 µl of the enzyme sample (MBP-ribAS) and 10 µl of H_2O resp. 10 µl of 100 mM pyruvaldehydoxim are added. The mixture is incubated for 1 h. A solution (50 µl) containing 2 % o-phenylenediamine, 20 mM DTT and 100 mM EDTA is added and the mixture incubated at 90°C for 1 h. The o-phenylenediamine reacts with 3,4-dihydroxy-2-butanone 4-phosphate to yield dimethylquinoxaline which is subsequently measured by reverse phase HPLC on a column of Nucleosil RP18. The eluent contains 100 mM ammonium formate and 25 % methanol. The effluent is monitored fluorometrically.

Screening Example 10

A part M containing 0 resp. 60 mM pyruvaldehydoxim in 20 µl H_2O is added to a mixture H containing 400 mM potassium phosphate pH 7.5, 50 mM $MgCl_2$ and 50 µg MBP_ribA enzyme in a total of 20 µl. A third solution J with 5mM ribulose-5-phosphate in 20 µl H_2O is added to start the reaction. The mixture is incubated for 30 min. at 37°C. A mixture P containing 50 mM DTT, 100 mM EDTA and 10 mM 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione in a total of 20 µl and a mixture R (20 µl) containing 200 mM potassium phosphate pH 7.5 and 10 U of 6,7- dimethyl-8-ribityl-lumazine synthase are added. The mixture is incubated for 1h at 37°C and then denaturated by adding 100 µl solution T containing 15% trichloroacetic acid. The amount of 6,7-dimethyl-8-ribityl-lumazine is determined fluorometrically (excitation 408 nm; emission 487 nm) by comparison with a 16 µM 6,7-dimethyl-8-ribityl-lumazine standard.

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Annex B

Description GTP cyclohydrolase II/3,4-dihydroxy-2-butanone-4-phosphate synthase

Organism Arabidopsis thaliana

```
          10         20         30         40         50         60
           |          |          |          |          |          |
ATGTCTTCCATCAATTTATCCTCATCATCTCCTTCCACCATCTCTCTTTCCCGCTCAAGA

 M  S  S  I  N  L  S  S  S  S  P  S  T  I  S  L  S  R  S  R

          70         80         90        100        110        120
           |          |          |          |          |          |
CTAAGCCAAAGCTCTACTACATTACTCCATGGACTACATCGTGTTACTTTACCATCTAAC

 L  S  Q  S  S  T  T  L  L  H  G  L  H  R  V  T  L  P  S  N

         130        140        150        160        170        180
           |          |          |          |          |          |
CATCCATTGTCAACCTTTTCCATTAAAACCAATACTGGAAAAGTTAAGGCTGCAGTGATC

 H  P  L  S  T  F  S  I  K  T  N  T  G  K  V  K  A  A  V  I

         190        200        210        220        230        240
           |          |          |          |          |          |
TCTAGAGAAGATGATCTGCTCTCATTCACCAACGGAAACACTCCTCTCTCAAATGGGTCT

 S  R  E  D  D  L  L  S  F  T  N  G  N  T  P  L  S  N  G  S

         250        260        270        280        290        300
           |          |          |          |          |          |
CTCATTGATGATCGGACCGAAGAGCCATTAGAGGCTGATTCGGTTTCACTTGGAACACTT

 L  I  D  D  R  T  E  E  P  L  E  A  D  S  V  S  L  G  T  L

         310        320        330        340        350        360
           |          |          |          |          |          |
GCTGCTGATTCTGCTCCTGCACCAGCCAATGGTTTTGTTGCTGAAGATGATGACTTTGAG

 A  A  D  S  A  P  A  P  A  N  G  F  V  A  E  D  D  D  F  E

         370        380        390        400        410        420
           |          |          |          |          |          |
TTGGATTTACCAACTCCTGGTTTCTCTTCTATCCCCGAGGCCATTGAAGATATACGCCAA

 L  D  L  P  T  P  G  F  S  S  I  P  E  A  I  E  D  I  R  Q
```

Annex B (cont.)

```
       430        440        450        460        470        480
        |          |          |          |          |          |
GGAAAGCTTGTGGTGGTTGTGGATGATGAAGATAGGGAAAATGAAGGGGATTTGGTGATG

 G  K  L  V  V  V  V  D  D  E  D  R  E  N  E  G  D  L  V  M

       490        500        510        520        530        540
        |          |          |          |          |          |
GCTGCTCAGTTAGCAACACCTGAAGCTATGGCTTTTATTGTGAGACATGGAACTGGGATA

 A  A  Q  L  A  T  P  E  A  M  A  F  I  V  R  H  G  T  G  I

       550        560        570        580        590        600
        |          |          |          |          |          |
GTTTGTGTGAGCATGAAAGAAGATGATCTCGAGAGGTTGCACCTTCCTCTAATGGTGAAT

 V  C  V  S  M  K  E  D  D  L  E  R  L  H  L  P  L  M  V  N

       610        620        630        640        650        660
        |          |          |          |          |          |
CAGAAGGAAAACGAAGAAAAGCTCTCTACTGCATTTACAGTGACTGTGGATGCAAAACAT

 Q  K  E  N  E  E  K  L  S  T  A  F  T  V  T  V  D  A  K  H

       670        680        690        700        710        720
        |          |          |          |          |          |
GGTACAACAACGGGAGTATCAGCTCGTGACAGGGCAACAACCATATTGTCTCTTGCATCA

 G  T  T  T  G  V  S  A  R  D  R  A  T  T  I  L  S  L  A  S

       730        740        750        760        770        780
        |          |          |          |          |          |
AGAGATTCAAAGCCTGAGGATTTCAATCGTCCAGGTCATATCTTCCCACTGAAGTATCGG

 R  D  S  K  P  E  D  F  N  R  P  G  H  I  F  P  L  K  Y  R

       790        800        810        820        830        840
        |          |          |          |          |          |
GAAGGTGGGGTTCTGAAAAGGGCTGGACACACTGAAGCATCTGTTGATCTCACTGTTTTA

 E  G  G  V  L  K  R  A  G  H  T  E  A  S  V  D  L  T  V  L

       850        860        870        880        890        900
        |          |          |          |          |          |
GCTGGACTGGATCCTGTTGGAGTACTTTGTGAAATTGTTGATGATGATGGTTCCATGGCT

 A  G  L  D  P  V  G  V  L  C  E  I  V  D  D  D  G  S  M  A
```

Annex B (cont.)

```
      910       920       930       940       950       960
       |         |         |         |         |         |
AGATTACCAAAACTTCGTGAATTTGCCGCCGAGAACAACCTGAAAGTTGTTTCCATCGCA

 R  L  P  K  L  R  E  F  A  A  E  N  N  L  K  V  V  S  I  A

      970       980       990      1000      1010      1020
       |         |         |         |         |         |
GATTTGATCAGGTATAGAAGAAAGAGAGATAAATTAGTGGAACGTGCTTCTGCGGCTCGG

 D  L  I  R  Y  R  R  K  R  D  K  L  V  E  R  A  S  A  A  R

     1030      1040      1050      1060      1070      1080
       |         |         |         |         |         |
ATCCCAACAATGTGGGGACCTTTCACTGCTTACTGCTATAGGTCCATATTAGACGGAATA

 I  P  T  M  W  G  P  F  T  A  Y  C  Y  R  S  I  L  D  G  I

     1090      1100      1110      1120      1130      1140
       |         |         |         |         |         |
GAGCACATAGCAATGGTTAAGGGTGAGATTGGTGACGGTCAAGACATTCTCGTGAGAGTT

 E  H  I  A  M  V  K  G  E  I  G  D  G  Q  D  I  L  V  R  V

     1150      1160      1170      1180      1190      1200
       |         |         |         |         |         |
CATTCTGAATGTCTAACAGGGGACATATTTGGGTCTGCAAGGTGTGATTGCGGGAACCAG

 H  S  E  C  L  T  G  D  I  F  G  S  A  R  C  D  C  G  N  Q

     1210      1220      1230      1240      1250      1260
       |         |         |         |         |         |
CTAGCACTCTCGATGCAGCAGATCGAGGCTACTGGTCGCGGTGTGCTGGTTTACCTACGT

 L  A  L  S  M  Q  Q  I  E  A  T  G  R  G  V  L  V  Y  L  R

     1270      1280      1290      1300      1310      1320
       |         |         |         |         |         |
GGACATGAAGGAAGAGGGATCGGTTTAGGACACAAGCTTCGAGCTTACAATCTGCAAGAT

 G  H  E  G  R  G  I  G  L  G  H  K  L  R  A  Y  N  L  Q  D

     1330      1340      1350      1360      1370      1380
       |         |         |         |         |         |
GCTGGTCGAGACACGGTTGAAGCTAATGAGGAATTAGGACTTCCTGTTGATTCTAGAGAG

 A  G  R  D  T  V  E  A  N  E  E  L  G  L  P  V  D  S  R  E
```

Annex B (cont.)

```
      1390       1400       1410       1420       1430       1440
        |          |          |          |          |          |
TATGGAATTGGTGCACAGATAATAAGGGATTTAGGTGTTAGGACAATGAAGCTGATGACA

 Y  G  I  G  A  Q  I  I  R  D  L  G  V  R  T  M  K  L  M  T

      1450       1460       1470       1480       1490       1500
        |          |          |          |          |          |
AATAATCCCGCAAAGTATGTTGGTTTGAAGGGATATGGATTAGCCATTGTTGGGAGAGTC

 N  N  P  A  K  Y  V  G  L  K  G  Y  G  L  A  I  V  G  R  V

      1510       1520       1530       1540       1550       1560
        |          |          |          |          |          |
CCTCTATTGAGTCTTATCACGAAGGAGAATAAGAGATATCTGGAGACAAAGCGGACCAAG

 P  L  L  S  L  I  T  K  E  N  K  R  Y  L  E  T  K  R  T  K

      1570       1580       1590       1600       1610       1620
        |          |          |          |          |          |
ATGGGTCACATGTATGGCTTGAAGTTCAAAGGGGATGTTGTGGAGAAGATTGAGTCTGAA

 M  G  H  M  Y  G  L  K  F  K  G  D  V  V  E  K  I  E  S  E

      1630
        |
TCTGAGTCCTAA

 S  E  S  -
```

Annex C

Description GTP cyclohydrolase II/3,4-dihydroxy-2-butanone-4-phosphate synthase

Organism Lycopersicon esculentum

```
          10         20         30         40         50         60
           |          |          |          |          |          |
ATGGCTTCCATCAACATTTCTTTCCCTTCAACAACATTTCACAGTCGATCACAAGCGAAC

 M  A  S  I  N  I  S  F  P  S  T  T  F  H  S  R  S  Q  A  N

          70         80         90        100        110        120
           |          |          |          |          |          |
TTCAAGTTGTTCAGTGGGTTGCATTCTGGAGATGTAGTTTCTCTTAATGGGGTCTCGTCA

 F  K  L  F  S  G  L  H  S  G  D  V  V  S  L  N  G  V  S  S

         130        140        150        160        170        180
           |          |          |          |          |          |
GGGTCATTCATACGGCTTAATGCTAGACCACACTTAACCATCAAGAGTGATTTCAAGATT

 G  S  F  I  R  L  N  A  R  P  H  L  T  I  K  S  D  F  K  I

         190        200        210        220        230        240
           |          |          |          |          |          |
ACTTCTGCATTGTTATCTGGTGAAGGAGACATCCGATTTCAATCCAGGGGAGAAAAAACT

 T  S  A  L  L  S  G  E  G  D  I  R  F  Q  S  R  G  E  K  T

         250        260        270        280        290        300
           |          |          |          |          |          |
CTGTTTAGTAGCCTCTCTACAGGAACTGAGACTCAACCTGATGCTGTAACCTTTGCAACG

 L  F  S  S  L  S  T  G  T  E  T  Q  P  D  A  V  T  F  A  T

         310        320        330        340        350        360
           |          |          |          |          |          |
CTAGAAGCAGATGGTGTTCCTACAACTAGTGGTTTTCTTTCAGATGACGATGAATGTGAT

 L  E  A  D  G  V  P  T  T  S  G  F  L  S  D  D  D  E  C  D

         370        380        390        400        410        420
           |          |          |          |          |          |
TTGGATCGACCGACCGAAGGTTTTTCATCTGTTCCTGAGGCTATTGAGGACATTCGCCAA

 L  D  R  P  T  E  G  F  S  S  V  P  E  A  I  E  D  I  R  Q

         430        440        450        460        470        480
```

Annex C (cont.)

```
        |         |         |         |         |         |
GGAAAGATGGTGCTAGTTACAGATGATGAAGACAGAGAAAATGAAGGGGATTTAGTTATG

 G  K  M  V  L  V  T  D  D  E  D  R  E  N  E  G  D  L  V  M

       490       500       510       520       530       540
        |         |         |         |         |         |
GCTGCATCCAAAGCTACACCAGAAGCAATGGCTTTCTTTGTGAAGTATGGAACAGGGATA

 A  A  S  K  A  T  P  E  A  M  A  F  F  V  K  Y  G  T  G  I

       550       560       570       580       590       600
        |         |         |         |         |         |
GTTTGTGTGAGCATGACAGAAGAACACTTGGAGAGGCTACAGCTTCCGTTGATGGTAAAC

 V  C  V  S  M  T  E  E  H  L  E  R  L  Q  L  P  L  M  V  N

       610       620       630       640       650       660
        |         |         |         |         |         |
GATAAAAAGAATGAGGAGAAACTTTGTACAGCATTCACAGTCTCAGTGGATGCGAAACAT

 D  K  K  N  E  E  K  L  C  T  A  F  T  V  S  V  D  A  K  H

       670       680       690       700       710       720
        |         |         |         |         |         |
GGAACGACTACAGGAGTATCTGCTCATGATAGAGCAACGACAGTATTGGCACTTGCGTCC

 G  T  T  T  G  V  S  A  H  D  R  A  T  T  V  L  A  L  A  S

       730       740       750       760       770       780
        |         |         |         |         |         |
GGAGATTCAAAGCCTGAGGATTTCAATCGACCGGGACATATCTTTCCTTTGAAATACAGG

 G  D  S  K  P  E  D  F  N  R  P  G  H  I  F  P  L  K  Y  R

       790       800       810       820       830       840
        |         |         |         |         |         |
GAAGGCGGGGTTTTAAAACGAGCTGGACATACTGAGGCTTCTGTGGATCTTGCCGTGTTA

 E  G  G  V  L  K  R  A  G  H  T  E  A  S  V  D  L  A  V  L

       850       860       870       880       890       900
        |         |         |         |         |         |
GCTGGCTTAGACCCTGTTGGAGTAATATGTGAGGTTGTGGATGATGATGGTTCCATGGCT

 A  G  L  D  P  V  G  V  I  C  E  V  V  D  D  D  G  S  M  A

       910       920       930       940       950       960
```

Annex C (cont.)

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                                                                    |
AGATTGCCTATGCTTCGTCAATTTGCAAAAGAACATAACTTGAAGATCATATCAGTTGCT

 R  L  P  M  L  R  Q  F  A  K  E  H  N  L  K  I  I  S  V  A

       970        980        990       1000       1010       1020
        |          |          |          |          |          |
GACTTAATCAGATATAGGAGAAAAACAGATCAGCTGGTAGAGCATGCTTCTGCTGCAAGA

 D  L  I  R  Y  R  R  K  T  D  Q  L  V  E  H  A  S  A  A  R

      1030       1040       1050       1060       1070       1080
        |          |          |          |          |          |
ATACCTACAATGTGGGGCCCGTTTACTGCCCACTGCTTCAAGTCAATAATAGATGGAATT

 I  P  T  M  W  G  P  F  T  A  H  C  F  K  S  I  I  D  G  I

      1090       1100       1110       1120       1130       1140
        |          |          |          |          |          |
GAGCACATTGCTATGGTTAAGGGGGATATAGGAGATGGACAAGATATTCTTGTTCGGGTA

 E  H  I  A  M  V  K  G  D  I  G  D  G  Q  D  I  L  V  R  V

      1150       1160       1170       1180       1190       1200
        |          |          |          |          |          |
CACTCGGAATGCCTCACAGGAGATATATTTGGTTCAGCCAGATGTGACTGTGGATCCCAG

 H  S  E  C  L  T  G  D  I  F  G  S  A  R  C  D  C  G  S  Q

      1210       1220       1230       1240       1250       1260
        |          |          |          |          |          |
CTGGCAACTGCAATGAAGCAAATTGAGGCTGCTGGCAGGGGGGTTTTGGTTTACCTCCGC

 L  A  T  A  M  K  Q  I  E  A  A  G  R  G  V  L  V  Y  L  R

      1270       1280       1290       1300       1310       1320
        |          |          |          |          |          |
GGTCATGATGGTAGAGGCATTGGTTTGGGTCACAAACTTCGTGCTTATAATTTACAAGAT

 G  H  D  G  R  G  I  G  L  G  H  K  L  R  A  Y  N  L  Q  D

      1330       1340       1350       1360       1370       1380
        |          |          |          |          |          |
GCTGGACGTGATACTGTTGAAGCGAATGAAGATCTTGGTTTGCCCGTTGATTCAAGAGAG

 A  G  R  D  T  V  E  A  N  E  D  L  G  L  P  V  D  S  R  E

      1390       1400       1410       1420       1430       1440
```

Annex C (cont.)

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          |         |         |         |         |         |
TATGGGGTTGGTGCACAGATCTTAAGGGATCTTGGTGTTCGAACTATGAAGTTTATAACG

 Y  G  V  G  A  Q  I  L  R  D  L  G  V  R  T  M  K  F  I  T

      1450      1460      1470      1480      1490      1500
         |         |         |         |         |         |
AACAATCCTGCACAATACAGCTGGCTAAAAGGTTATGGTTTGGCAATTTCTGGTATGGTC

 N  N  P  A  Q  Y  S  W  L  K  G  Y  G  L  A  I  S  G  M  V

      1510      1520      1530      1540      1550      1560
         |         |         |         |         |         |
CCCGTTGTTACTCCCTTTACTAACCACTACGACACATATTTGGAAACAACACGAGCTAAA

 P  V  V  T  P  F  T  N  H  Y  D  T  Y  L  E  T  T  R  A  K

      1570      1580      1590      1600      1610      1620
         |         |         |         |         |         |
ATGGGCCATGTATACGGCTTGAACCTTATTCGCCCGGCAACCAGCACCAGCACAACAAAT

 M  G  H  V  Y  G  L  N  L  I  R  P  A  T  S  T  S  T  T  N

      1630      1640      1650
         |         |         |
GGTAAACCAAA=TCTGAGAATACTTCTACTATAAGATGA

 G  K  P  N  S  E  N  T  S  T  I  R  -
```

Annex D

Description 2,5-diamino-6-ribosylamino-4(3H)-pyrimidinone reductase

Organism Saccharomyces cerevisiae

```
          10         20         30         40         50         60
           |          |          |          |          |          |
ATGTCTTTGACACCACTGTGTGAAGATTTACCACAATTTCTGCAAAACTATCTACCGAAT

 M  S  L  T  P  L  C  E  D  L  P  Q  F  L  Q  N  Y  L  P  N

          70         80         90        100        110        120
           |          |          |          |          |          |
GCTGGTCAAACGGAAAATACCATTGTGCCCTTTGTCACACTAACTTATGCTCAATCGCTC

 A  G  Q  T  E  N  T  I  V  P  F  V  T  L  T  Y  A  Q  S  L

         130        140        150        160        170        180
           |          |          |          |          |          |
GACGCGAGAGTATCTAGGGGCCCTGGAGTGAGGACTACAATTTCACATCCCGAGACCAAA

 D  A  R  V  S  R  G  P  G  V  R  T  T  I  S  H  P  E  T  K

         190        200        210        220        230        240
           |          |          |          |          |          |
ACAATGACGCATTATTTGAGACATCATCACGATGGAATACTCGTAGGAAGTGGAACAGTG

 T  M  T  H  Y  L  R  H  H  H  D  G  I  L  V  G  S  G  T  V

         250        260        270        280        290        300
           |          |          |          |          |          |
CTAGCTGATAATCCTGGATTGAATTGTAAATGGGGTCCCGATCCGGCTGCAAATTCCCCA

 L  A  D  N  P  G  L  N  C  K  W  G  P  D  P  A  A  N  S  P

         310        320        330        340        350        360
           |          |          |          |          |          |
AGGCCAATAATAATAGATACAAAGCAAAAGTGGCGATTTGATGGTTCAAAAATGCAAGAA

 R  P  I  I  I  D  T  K  Q  K  W  R  F  D  G  S  K  M  Q  E

         370        380        390        400        410        420
           |          |          |          |          |          |
CTTTTTATTAAACGACAGGGTAAGCCGCCAATCGTTGTTGTCACAAGTGAGCCCATTATA

 L  F  I  K  R  Q  G  K  P  P  I  V  V  V  T  S  E  P  I  I

         430        440        450        460        470        480
           |          |          |          |          |          |
```

Annex D (cont.)

```
AAAGAACAACATGTAGACTACGCAATTTGTCCAATAAATGATACTACGAAATTGGTCGAT
 K  E  Q  H  V  D  Y  A  I  C  P  I  N  D  T  T  K  L  V  D

       490       500       510       520       530       540
        |         |         |         |         |         |
TGGAAGAAATTGTTTGAGATATTAAAAGAAGAATTCAATATAAGGTCAGTAATGGTTGAA
 W  K  K  L  F  E  I  L  K  E  E  F  N  I  R  S  V  M  V  E

       550       560       570       580       590       600
        |         |         |         |         |         |
GGAGGTGCCAATGTAATAAATCAGTTGTTGCTGAGGAGCGATATTGTCAACAGTCTTATA
 G  G  A  N  V  I  N  Q  L  L  L  R  S  D  I  V  N  S  L  I

       610       620       630       640       650       660
        |         |         |         |         |         |
ATAACTATTGGATCAACATTTCTGGGTAGCTCAGGCACCGAAGTTAGCCCACCCCAAACA
 I  T  I  G  S  T  F  L  G  S  S  G  T  E  V  S  P  P  Q  T

       670       680       690       700       710       720
        |         |         |         |         |         |
GTAAATTTAAAGGATATGTCATGGTGGAAGGGCATTACCGATGTGGTGCTTTGTGCGAGA
 V  N  L  K  D  M  S  W  W  K  G  I  T  D  V  V  L  C  A  R

       730
        |
CTGGCCGATGACTAA
 L  A  D  D  -
```

Claims

1. A method for screening for the presence or absence of inhibition of GTP cyclohydrolase II activity, comprising the following steps:

(a) preparing a first aqueous mixture containing a protein having a GTP cyclohydrolase II sequence and GTP,

(b) reacting said first mixture during a predetermined period of time at a predetermined temperature and subsequently detecting the level of 2,5-diamino-6-ribosylamino-4(3H)-pyrimidinone 5'-phosphate,

(c) preparing a second aqueous mixture by including in said first mixture a predetermined amount of a chemical test sample

(d) reacting said second mixture during the predetermined period of time at the predetermined temperature and subsequently detecting the level of 2,5-diamino-6-ribosylamino-4(3H)-pyrimidinone 5'-phosphate,

(e) determining the presence of inhibition of GTP cyclohydrolase II by observation of whether the level detected in step (d) is lower than the level detected in step (b).

2. A method for screening for the presence or absence of inhibition of GTP cyclohydrolase II activity, comprising the following steps:

(a) preparing a first aqueous mixture containing a protein having a mutated plant-type GTP cyclohydrolase II sequence and GTP,

(b) reacting said first mixture during a predetermined period of time at a predetermined temperature and subsequently detecting the level of 2,5-diamino-6-ribosylamino-4(3H)-pyrimidinone 5'-phosphate,

(c) preparing a second aqueous mixture by including in said first mixture a predetermined amount of a specific inhibitor for said GTP cyclohydrolase II,

(d) reacting said second mixture during the predetermined period of time at the predetermined temperature and subsequently

detecting the level of 2,5-diamino-6-ribosylamino-4(3H)-pyrimidinone 5'-phosphate,

(e) determining the presence of resistance to inhibition of GTP cyclohydrolase II by observation of whether the level detected in step (d) is similar to the level detected in step (b).

3. The method according to claims 1 or 2, wherein said aqueous mixture contains a bivalent metal ion.

4. The method according to claim 3, wherein said bivalent metal ion is the magnesium ion.

5. The method according to claims 1 or 2, wherein said mixture contains an antioxidative substance.

6. The method according to claims 1 or 2, wherein said mixture has a pH in the range of 6 to 9.5.

7. The method according to claims 1 or 2, wherein a premixture is prepared which lacks one essential ingredient and the reaction is started by adding said ingredient.

8. The method according to claims 1 or 2 characterized in that the level of 2,5-diamino-6-ribosylamino-4(3H)-pyrimidinon 5'-phosphate is detected directly or after chemical or enzymatic derivatization.

9. The method according to claims 1 and 8 or 2 and 8, wherein said direct detection is carried out after the reaction has been terminated by adding a chelating agent for said bivalent metal ion.

10. The method according to claims 1 or 2, wherein the chemical derivatization is carried out by adding a vicinal dioxo compound.

11. The method according to claim 10, wherein said dioxo compound is

diacetyl.

12. The method according to claims 1 or 2, wherein the enzymatic detection is carried out by adding 2,5-diamino-6-ribosylamino-4(3H)-pyrimidinone 5'-phosphate reductase and NAD(P)H and detecting the formation of NAD(P).

13. A method for screening for the presence or absence of inhibition of 3,4-dihydroxy-2-butanone 4-phosphate synthase activity, comprising the following steps:

(a) preparing a first aqueous mixture containing a protein having a 3,4-dihydroxy-2-butanone 4-phosphate synthase sequence and ribulose 5-phosphate,

(b) reacting said mixture during a predetermined period of time at a predetermined temperature and subsequently detecting the level of 3,4-dihydroxy-2-butanone 4-phosphate,

(c) preparing a second aqueous mixture by including in said first mixture a predetermined amount of a specific inhibitor for said 3,4-dihydroxy-2-butanone 4-phosphate synthase,

(d) reacting said second mixture during the predetermined period of time at the predetermined temperature and subsequently detecting again the level of 3,4-dihydroxy-2-butanone 4-phosphate,

(e) determining the presence of inhibition of 3,4-dihydroxy-2-butanone 4-phosphate synthase by observation of whether the level detected in step (d) is lower than the level detected in step (b).

14. A method for screening for the presence or absence of inhibition of 3,4-dihydroxy-2-butanone 4-phosphate synthase activity, comprising the following steps:

(a) preparing a first aqueous mixture containing a protein having a mutated plant-type 3,4-dihydroxy-2-butanone 4-phosphate synthase sequence and ribulose-5-phosphate,

(b) reacting said mixture during a predetermined period of time at a predetermined temperature and subsequently detecting the level of 3,4-dihydroxy-2-butanone 4-phosphate,

(c) preparing a second aqueous mixture by including in said first mixture a predetermined amount of a specific inhibitor for said 3,4-dihydroxy-2-butanone 4-phosphate synthase,

(d) reacting said second mixture during the predetermined period of time at the predetermined temperature and subsequently detecting again the level of 3,4-dihydroxy-2-butanone 4-phosphate,

(e) determining the presence of resistance to inhibition of 3,4-dihydroxy-2-butanone 4-phosphate synthase by observation of whether the level detected in step (d) is similar to the level detected in step (b).

15. The method according to claims 13 or 14, wherein said aqueous mixture contains a bivalent metal ion.

16. The method according to claim 15, wherein said bivalent metal ion is the magnesium ion.

17. The method according to claims 13 or 14, wherein said mixture has a pH in the range of 6 to 9.5.

18. The method according to claims 13 or 14, wherein a premixture is prepared which lacks one essential ingredient and the reaction is started by adding said ingredient.

19. The method according to claims 13 or 14, wherein the presence of 3,4-dihydroxy-2-butanone 4-phosphate is detected directly or after chemical or enzymatic derivatization.

20. The method according to claim 19, wherein said detection is carried out after the reaction has been terminated by adding a chelating agent for

said bivalent metal ion.

21. The method according to claim 19, wherein said chemical derivatization is carried out by adding an aromatic or heteroaromatic ortho-diamine.

22. The method according to claim 20, wherein for enzymatic derivatization 6,7-dimethyl-8-ribityllumazine synthase and 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione are added and the level of 6,7-dimethyl-8-ribityl-lumazine is detected.

23. The method according to claim 20, wherein for enzymatic derivatization 6,7-dimethyl-8-ribityllumazine synthase, riboflavin synthase and 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione are added and the level of riboflavin is detected.

24. The method according to claims 13 or 14 characterized in that said first aqueous mixture is prepared by
pre-reacting a first pre-mixture containing pentose-phosphate isomerase and ribose 5-phosphate and
mixing said pre-reacted first pre-mixture with a second pre-mixture containing
said protein having the 3,4-dihydroxy-2-butanone 4-phosphate synthase sequence or
said protein having said mutated 3,4-dihydroxy-2-butanone 4-phosphate synthase sequence.

25. Kit of parts for screening for the presence or absence of inhibition of GTP cyclohydrolase II activity in a chemical test sample, comprising a part A with a protein having a GTP cyclohydrolase II sequence, and a part B with GTP.

26. Kit of parts for screening for the presence or absence of resistance of a protein having a mutated GTP cyclohydrolase II sequence to a specific inhibitor for GTP cyclohydrolase II activity comprising

a part C with said specific inhibitor, and
a part B with GTP.

27. Kit of parts according to one of claims 25 or 26, additionally comprising a bivalent metal ion in at least one of parts A, B or C or in a separate part D.

28. Kit of parts according to claim 27 wherein a chelating agent for said bivalent metal ion is contained in a separate part E.

29. Kit of parts according to one of claims 25 to 28 additionally comprising a vicinal dioxo compound either in part E or in a separate part F.

30. Kit of parts according to one of claims 25 to 28 additionally comprising 2,5-diamino-6-ribosylamino-4(3H)-pyrimidinone 5'-phosphate reductase in at least one of parts A, B, C, D, E and/or a separate part G; and NAD(P)H in at least one of part A, B, C, D, E, G.

31. Kit of parts for screening for the presence or absence of inhibition of 3,4-dihydroxy-2-butanone 4-phosphate synthase activity in a chemical test sample, comprising
a part H with a protein having a 3,4-dihydroxy-2-butanone 4-phosphate synthase sequence and either
a part J with ribulose 5-phosphate or
a part K with pentose-phosphate isomerase in conjunction with a part L with ribose 5-phosphate.

32. Kit of parts for screening for the presence or absence of resistance of a protein having a mutated 3,4-dihydroxy-2-butanone 4-phosphate synthase sequence to a specific inhibitor for 3,4-dihydroxy-2-butanone 4-phosphate synthase activity comprising
a part M with said specific inhibitor and either
a part J with ribulose 5-phosphate or a part K with pentose-phosphate isomerase in conjunction with a part L with ribose 5-phosphate.

33. Kit of parts according to one of claims 31 to 32, additionally comprising a bivalent metal ion in at least one of parts H, J, K, L, M or in a separate part N.

34. Kit of parts according to claim 33, wherein a chelating agent for said bivalent metal ion is contained in a separate part P.

35. Kit of parts according to one of claims 31 to 34 additionally comprising an aromatic or heteroaromatic ortho-diamine either in part P or in a separate part Q.

36. Kit of parts according to one of claims 31 to 34, additionally comprising 6,7-dimethyl-8-ribityllumazine synthase in at least one of parts H, J, K, L, M, N, P or in a separate part R and 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione in at least one of parts H, J, K, L, M, N, P, R.

37. Kit of parts according to claim 36 comprising additionally riboflavin synthase in at least one of parts H, J, K, L, M, N, P, R or in a separate part S.

38. Isolated protein having a plant enzyme sequence of GTP cyclohydrolase II and/or 3,4-dihydroxy-2-butanone 4-phosphate synthase and having the function of GTP cyclohydrolase and/or 3,4-dihydroxy-2-butanone-4-phosphate synthase.

39. Protein according to claim 38 having additionally an auxiliary protein sequence.

40. Protein according to claim 38 wherein the plant is a monocotyledonous or dicotyledonous plant.

41. Isolated DNA coding exclusively for a protein in accordance with claim 38 and optionally at least one other enzyme of the flavin biosynthesis

pathways.

42. A vector comprising the nucleotide sequence of the DNA in accordance with claim 41.

43. A method of inhibiting an enzyme with GTP cyclohydrolase II activity and 3,4-dihydroxy-2-butanone 4-phosphate synthase activity of or in a plant by treatment with a compound selected from the group of chemical compounds that exhibit inhibition in the method of claims 1 or 13.

44. A method of inhibiting an enzyme with GTP cyclohydrolase II activity of or in a microorganism by treatment with a compound selected from the group of chemical compounds that exhibit inhibition in the method of claim 1.

45. A method of inhibiting an enzyme with 3,4-dihydroxy-2-butanone 4-phosphate synthase activity of or in a microorganism by treatment with a compound selected from the group of chemical compounds exhibiting inhibition in the method of claim 13.

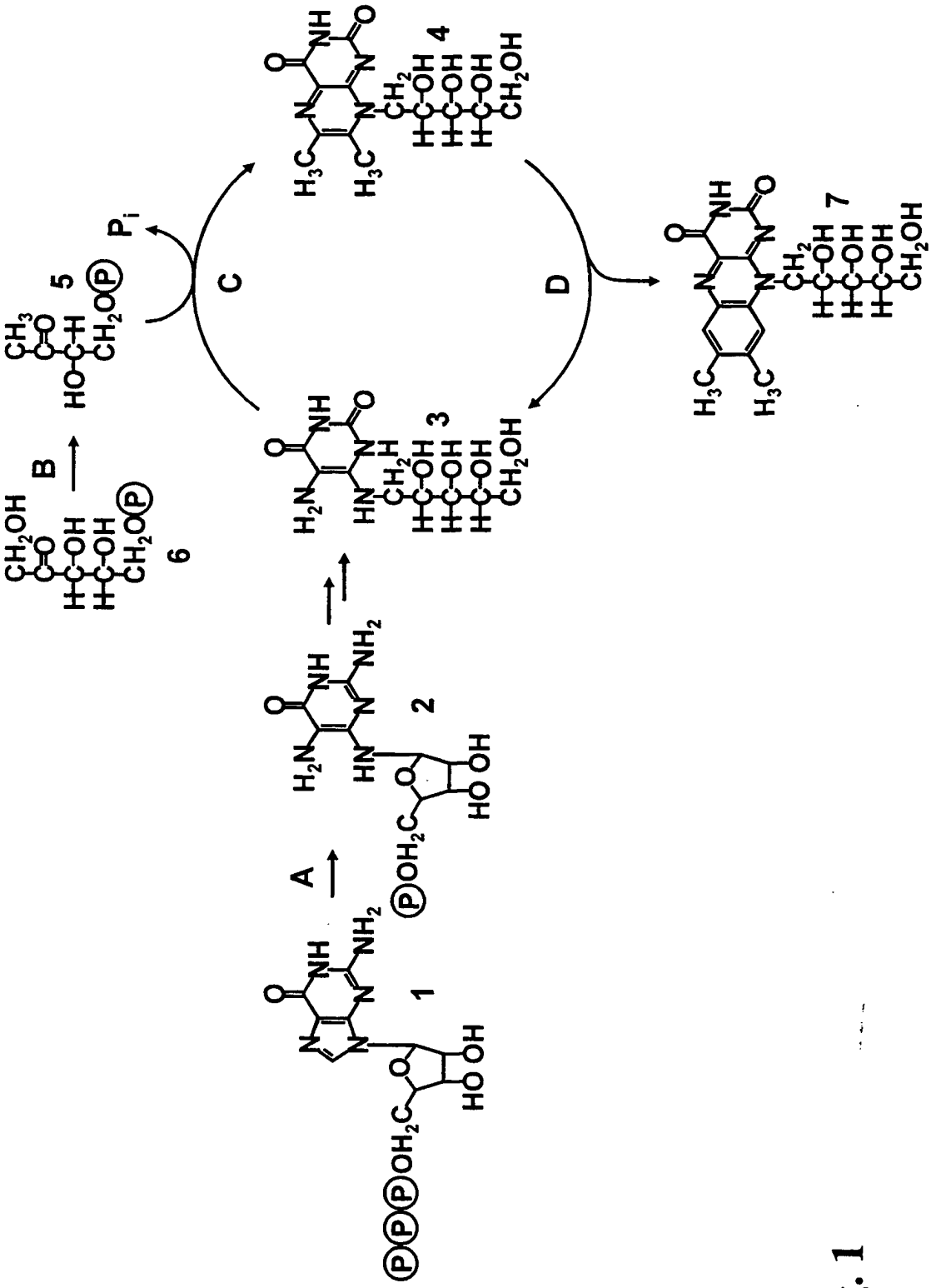


Fig. 1

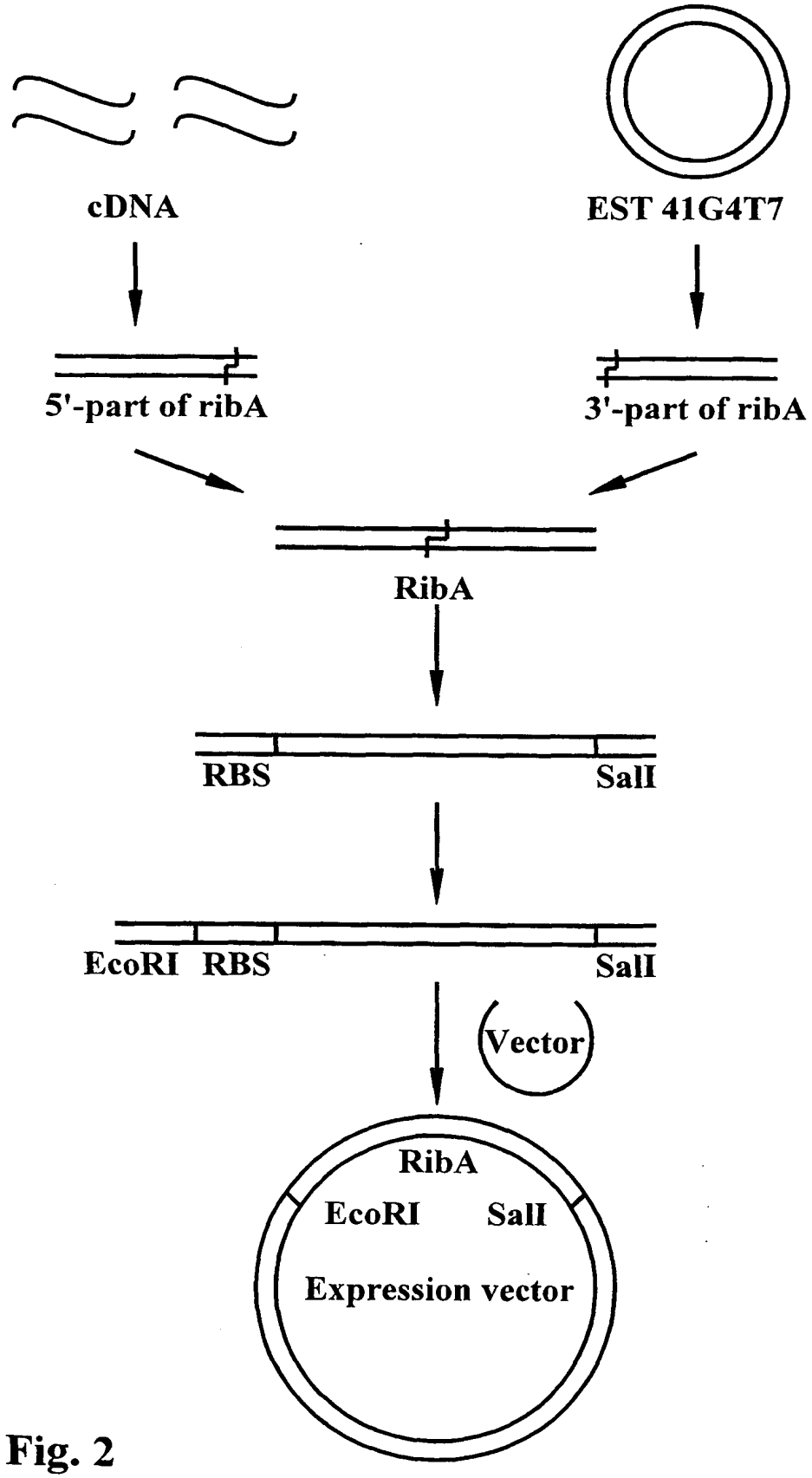
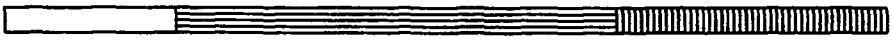


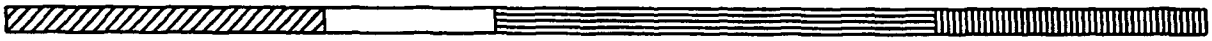
Fig. 2

(a) S-ribA



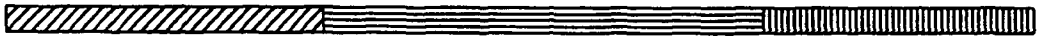
signal sequence | 3,4-dihydroxy-2-butanone-4 phosphate synthase | GTP-cyclohydrolase II

(b) malE-S-ribA



maltose binding protein | signal sequence | 3,4-dihydroxy-2-butanone-4 phosphate synthase | GTP-cyclohydrolase II

(c) malE-ribA



maltose binding protein | 3,4-dihydroxy-2-butanone-4 phosphate synthase | GTP-cyclohydrolase II

Fig. 3

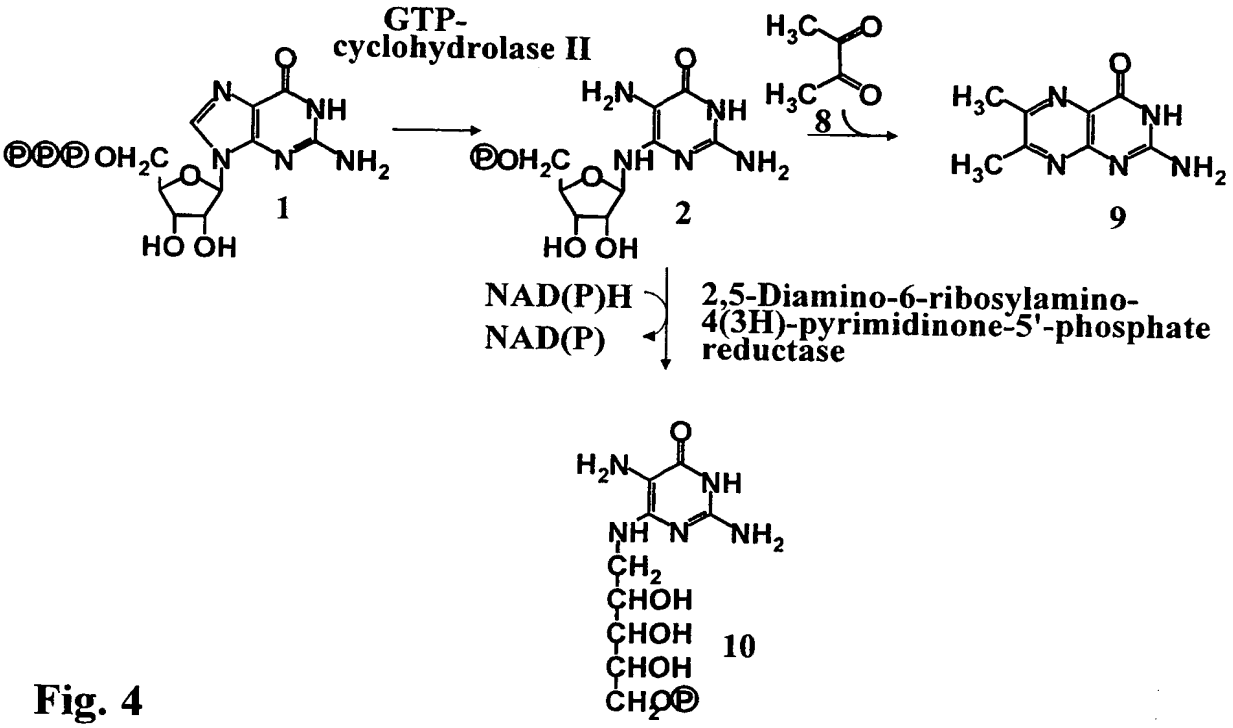


Fig. 4

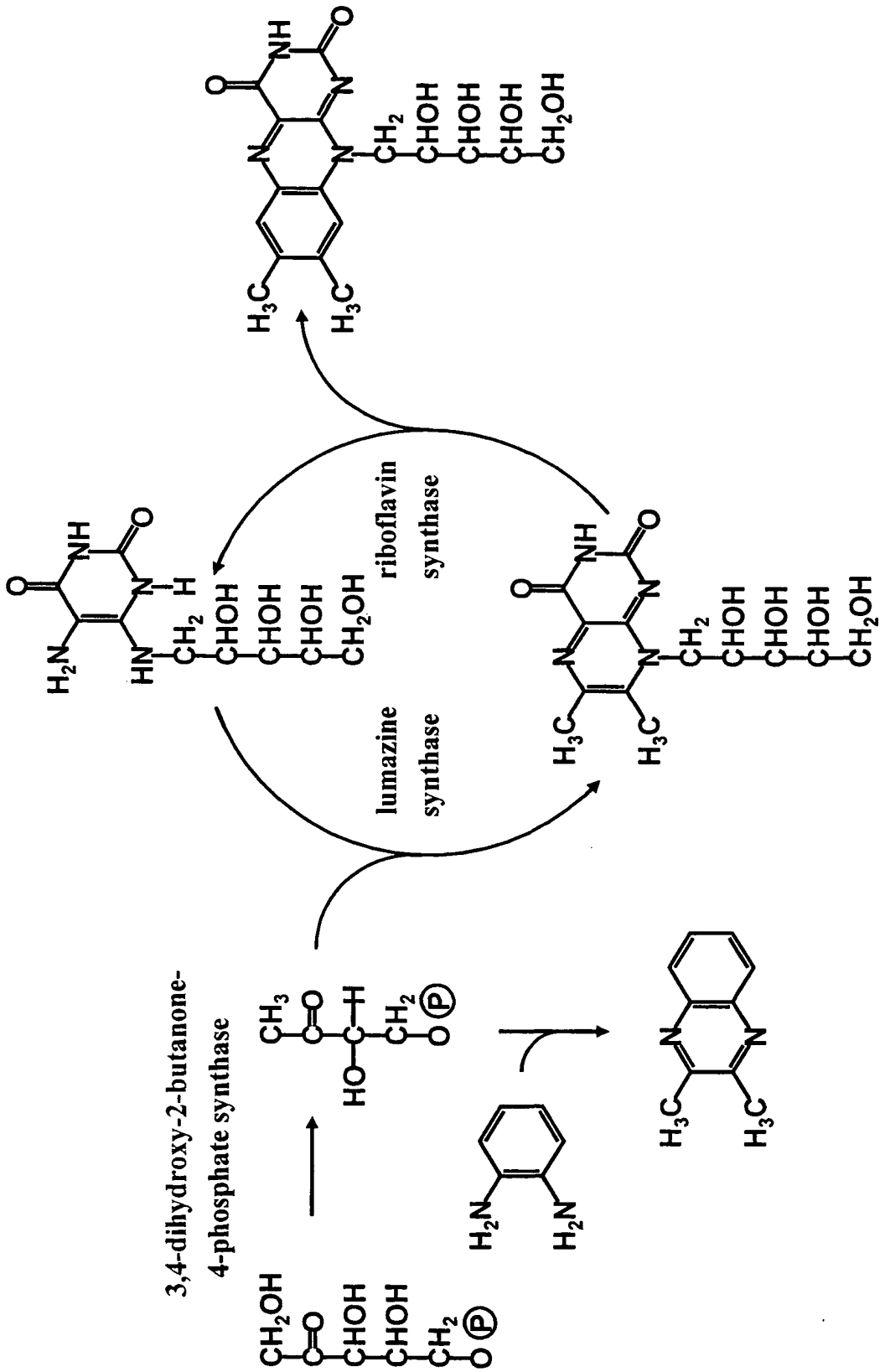


Fig. 5

INTERNATIONAL SEARCH REPORT

Inte. .ional Application No
PCT/EP 99/09936

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12Q1/34 C12Q1/25 C12N9/78

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE EMBL SEQ. DATA LIBRAR EMBL, Heidelberg, Germany Accession Number AJ000053, 1 October 1997 (1997-10-01) HERZ, S.W., ET AL.: "Biosynthesis of riboflavin in plants. The ribA gene of Arabidopsis thaliana specifies a bifunctional GTP cyclohydrolase II/3,4-dihydroxy-2-butanone-4-phosphate synthase." XP002110950 the whole document --- -/--	38-42

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
31 May 2000	09/06/2000

Name and mailing address of the ISA	Authorized officer
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Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inte. ..ional Application No
PCT/EP 99/09936

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE EMBL SEQ. DATA LIBRAR EMBL, Heidelberg, Germany Accession Number AJ002298, 29 October 1997 (1997-10-29) HERZ, S.W., ET AL.: "Lycopersicon esculentum mRNA for GTP cyclohydrolase II/3,4-dihydroxy-2-butanone 4-phosphate synthase" XP002110951 the whole document	38-42
P,X	WO 99 38986 A (NOVARTIS ERFINDUNGEN VERWALTUN ;NOVARTIS AG (CH); GUYER CHARLES DA) 5 August 1999 (1999-08-05) page 11, line 11, paragraph 2 -page 12, line 22, paragraph 2	1-42
E	EP 0 967 281 A (DU PONT) 29 December 1999 (1999-12-29) page 16, line 45, paragraph 105 -page 17, line 3; claims 16-18; example 7	1-42
P,A	WO 99 27125 A (SMITHKLINE BEECHAM CORP) 3 June 1999 (1999-06-03) claims 14,20	1-42
A	RICHTER, G. ET AL: "Biosynthesis of riboflavin: 3,4- dihydroxy -2- butanone -4-phosphate synthase" METHODS ENZYMOL. (1997), 280(VITAMINS AND COENZYMES, PART J), 374-382 , XP000909099 page 377	1-7,9, 13-18, 20,22-37

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP 99/ 09936

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 43-45

Present claims 43-45 relate to the use of a compound defined with respect to a desirable property, namely its ability to inhibit the activity of enzymes involved in riboflavin biosynthesis, either GTP cyclohydrolase II (screening method of claim 1) or 3,4-dihydroxy-2-butanone 4-phosphate synthase (screening method of claim 13).

No technical features of the substances are present in claims 43-45 which would lead to such desirable properties, the technical features formulated so as to permit the execution of a meaningful search. Support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT is to be found for only a very small proportion of the substances which could fall within the scope of these claims, such as pyrophosphate (see page 29, table 1) or pyruvaldehyde oxime (page 32, table 2), shown in the description to act as inhibitors in the screening assays of the present application. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible Independent of the above reasoning, the claims also lack clarity (Article 6 PCT). An attempt is made to define the compound by reference to a result to be achieved. Again, this lack of clarity in the present case is such as to render a meaningful search over the whole of the claimed scope impossible.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter ..onal Application No
PCT/EP 99/09936

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9938986 A	05-08-1999	NONE	
EP 0967281 A	29-12-1999	NONE	
WO 9927125 A	03-06-1999	WO 9927126 A	03-06-1999